UNIVERSITY OF ALBERTA

OLFACTORY, BEHAVIORAL, AND ENDOCRINE RESPONSES TO PUTATIVE STEROIDAL PHEROMONES IN AN AFRICAN CICHLID (*HAPLOCHROMIS BURTONI*)

BY

TODD COLE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: OLFACTORY. BEHAVIORAL, AND ENDOCRINE RESPONSES TO PUTATIVE STEROIDAL PHEROMONES IN AN AFRICAN CICHLID, *HAPLOCHROMIS BURTONI* submitted by Todd Cole in partial fulfillment of the requirements for the degree of Master of Science in Environmental Biology & Ecology.

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Abstract

In many fish, steroid and prostaglandin hormones released by conspecifics are detected by the olfactory system and induce a variety of behavioral and physiological responses. Despite evidence that a number of freshwater fish have evolved such *hormonal pheromone* systems, little is known about the pheromones of the otherwise well-studied cichlid fishes (Family *Cichlidae*; Order *Perciformes*). Cichlids appear to be ideal subjects for investigating hormonal pheromone function because they readily reproduce in captivity, their rich behavioral repertoires should facilitate the development of behavioral pheromone bioassays, and their biodiversity and varied reproductive tactics provide numerous opportunities for comparative studies. Electro-olfactogram (EOG) studies using more than 160 synthetic steroids and prostaglandins show that an African rift lake cichlid, *Haplochromis burtoni*, does not detect prostaglandins but detects a variety of conjugated (sulfated or glucuronidated) 18-, 19-, and 21-carbon steroids with great specificity and sensitivity (0.01-1 nM olfactory detection threshold). EOG cross-adaptation and binary mixture experiments indicate that these steroids act through five independent receptor mechanisms. Behavioral bioassays involving exposure to single steroid odorants suggest that some of these detected steroids induce behavioral responses. Endocrine bioassay involving exposure to a mixture containing steroids acting on all five olfactory receptor types (testosterone-sulfate; estradiol-17-glucuronide; estradiol-3,17-disulfate; dehydroepiandrosterone-sulfate; 5β-pregnan-3α,17α-diol-20-one-3-glucuronide) significantly increased male serum testosterone levels within 60 min. Experiments to identify the active steroid(s) were unsuccessful. These results are the first to

demonstrate hormonal pheromone activity in *Haplochromis*, and suggest hormonal pheromones could provide novel approaches for examining the explosive speciation of African rift lake cichlids.

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1. Introduction

Pheromones are intraspecific chemical signals that elicit behavioral and/or physiological responses (Sorensen and Wyatt, 2000). Pheromones influence key aspects of fish behavior, including individual recognition, schooling, homing, parental and offspring recognition, predator avoidance, and reproduction (Liley, 1982; Sorensen and Stacey, 1999). Recently, it has been demonstrated that hormones (steroids and prostaglandins) and their metabolites serve as reproductive pheromones eliciting both courtship behavior and spawning synchrony in fish (Sorensen and Stacey, 1999). Pioneering work from Tavolga (1956) emphasized the importance of chemical signals in reproductive behavior, and Colombo et al. (1980) provided the first clear evidence for the importance of hormonal pheromones in fish. Since then, a number of studies have investigated the use of sex hormones as pheromones in a diverse array of fish species. To date, evidence for hormonal pheromones has been collected for nine orders of teleost fish (Stacey and Sorensen, 2002), with the majority of studies focusing on ostariophysin species from the order Cypriniformes (Sorensen and Stacey, 1999) and Siluriformes (Resink et al., 1989a). Studies of four species in particular have yielded compelling evidence that released hormones function as sex pheromones.

The African catfish (*Clarias gariepinus*) is thought to utilize hormonal pheromones to synchronize ovulation and facilitate spawning synchrony (Resink et al., 1989a). Olfactory stimuli are involved in ovulation induction and a combination of steroid glucuronides present in the seminal vesicle attract females to signaling males shortly before spawning (Resink et al., 1989a, b). More preliminary evidence suggests possible sources of pheromones in postovulatory ovarian fluid of females (Resink et al., 1989a) and the skin of males (Ali et al., 1987). Electro-olfactogram (EOG) studies, which examine olfactory responsiveness to steroid and prostaglandin compounds in an attempt to gain evidence for use of hormonal pheromones, have so far only been used to determine olfactory sensitivity to those steroid glucuronides produced in the seminal vesicle of *C. gariepinus* (Resink et al., 1989a).

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cichlids feasible. Finally, several studies have suggested that cichlids use pheromones.

The Mozambique tilapia (*Oreochromis mossambicus*) has a mating system similar to that of *H. burtoni*. Chemical analysis of male holding water and body fluids has implicated sulfated steroids as potent odorants in the urine, suggesting the importance of steroid hormones in the chemical communication of *O. mossambicus* (Frade et al., 2002). More recent studies suggest that male urination rates and courtship behavior increase in the presence of preovulatory females, and less dramatically in the presence of postovulatory females (Almeida et al., 2003). Further, female *O. mossambicus* exhibit large EOG responses to male urine fractions that included sulfated steroids, but not to fractions including non-sulfated steroids (Frade et al. 2003). However, EOG studies failed to show olfactory sensitivity to a number of tested steroids known to act as pheromones in other species (Frade et al., 2002). These results suggest that males can determine female reproductive status, and that sulfated steroids released in the urine of males play an important role in reproductive communication. Olfactory inhibition associated with an endosulfan insecticide has been shown to influence the reproductive behavior of *O. mossambicus* (Matthiessen and Logan, 1984), implying that chemical communication is a vital component of cichlid reproduction.

Chemical cues also appear to influence *H. burtoni* reproductive behavior. Crapon de Caprona (1974) showed that male *H. burtoni* exposed to water from the tank of a gravid female will exhibit an increase in courting, leading of females to spawning sites, and appearance of a visible aggressive signal (black eye bar). These results show a clear influence of conspecific chemical stimuli on the behavior of male *H. burtoni*, but provide no explanation of potential mechanisms of detection nor of specific compounds that elicit these responses. A more recent study (Robison et al., 1998) has indicated that *H. burtoni* detect at least 13 steroid hormones, two of which (5β-pregnan-3α,17-diol-20-one glucuronide and etiocholanolone-glucuronide) may have potent effects on the reproductive behavior of an African catfish (*Clarias gariepinus*) (Resink et al., 1989a). However, the findings of Robison et al. (1998)

were quite preliminary and the biological functions of the detected steroids were not studied .

H. burtoni appears to be an ideal cichlid in which to study the biological effects of hormonal pheromones. *H. burtoni* is endemic to Lake Tanganyika (Fernald and Hirata, 1977a), the second deepest and seventh largest lake in the world. This species has been studied extensively under both natural and semi-natural conditions, and its behavioral repertoire has been well documented (Heiligenberg, 1973; Fernald, 1977; Fernald and Hirata, 1977a; Francis et al., 1992; Mosler, 1985). As well, *H. burtoni* has been the subject of numerous studies including those on eye function (Fernald, 1985a; Allen and Fernald, 1985), eye growth (Kroeger and Fernald, 1994; Fernald, 1985b), eye development (Kroeger et al., 1994; Mack and Fernald, 1992), GnRH containing neurons (White and Fernald, 1993; Soma et al., 1996), development (Hofmann et al., 1999), olfactory communication (Crapon de Caprona, 1980; Robison et al., 1998), sound production (Nelissen, 1977), social regulation of sex and size (Fernald, 2002), and natural habitat (Fernald and Hirata, 1977b).

H. burtoni breeds readily under laboratory conditions and, as a maternal mouthbrooder, has a mating system similar to the majority of all known cichlid species (Staeck and Linke, 1996). *H. burtoni* shows a high degree of sexual dimorphism, with brightly colored males being significantly larger than uniformly gray females. Territorial males establish spawning sites that are typically a cleared piece of rock or a shallow pit dug in the substrate. After a brief display period that consists of the male quivering his body sideways in front of the female, the male attempts to lead the female back to his spawning site. If the female is receptive, she follows the male to his spawning site, releases her eggs and immediately begins to pick them up in her mouth. The male then extends his anal fin to expose egg spots as the female picks up her eggs. When the female attempts to pick up these false eggs, sperm are released and fertilization occurs in the mouth. Egg spots on the male's anal fin appear to be an important visual stimulus that facilitates egg fertilization in other haplochromine species (e.g. *H. desfonainesii*; e.g. Kirchshofler (1953). After fertilization, the female broods the clutch in her mouth for 2-3 weeks, and the fry reach sexual maturity approximately 3 months after release.

There is ample opportunity for hormonal pheromones to play a role in this mating system. Males do not attempt to court every passing female, and not all females are receptive to male courtship (Fernald, 1977). Further, not all females that are lead back to a spawning site will release their eggs. Thus, there is opportunity at the point of encounter and at the spawning site itself for mate choice that is chemically based. Males are under constant pressure to recognize periovulatory females because time spent chasing and courting females allows floater males to enter and/or claim the vacant territory. It would be beneficial for males to have a means of assessing the reproductive status of females during an encounter. Water-borne hormones, released into water, could provide a means by which males evaluate female reproductive status. One might also expect *H. burtoni* to have evolved a chemical communication system given the turbid nature of their natural habitat (Fernald and Hirata, 1977b).

Lake Tanganyika is a unique ecosystem because it has evolved for the most part in complete isolation from other water systems (Fernald and Hirata, 1977). The three east African great lakes (Tanganyika, Malawi, and Victoria) each contain no less than 250 endemic species of cichlids, and their amazing variety has arisen within the past one million years (Stiassny and Meyer, 1999). Lake Victoria contains a monophyletic group of 300 known cichlids that are thought to have evolved within the past 14,000 years (Stiassny and Meyer, 1999; Seehausen, 2002). Because of this explosive speciation, cichlids have been the subject of many evolutionary and ecological studies (Meyer et al., 1990; Meyer, 1993; Takahashi et al., 2001; Turner et al. 2001). Further, several studies have concluded that haplochromines may have been important seed species in early Tanganyikan cichlid speciation (Sturmbauer and Meyer, 1993; Mayer et al., 1998; Salzburger et al., 2002). Olfactory cues have been implicated in other fish (e.g. Sakai and Yoshii, 1990) as a potential means of achieving species-specific chemical communication. Recognition of species-specific stimuli during aggressive encounters has been documented in *H. burtoni* (Heiligenberg and Kramer, 1972) suggesting that *H. burtoni* may be an excellent model species to examine the possibility of pheromones to influence rapid speciation.

Cichlids have also been studied because of the recent decline of cichlid populations. Cichlid fishes represent the largest familial group of endangered

vertebrates (Reid, 1990). Introduction of the nile perch (*Lates niloticus*) to the African rift lake system combined with overfishing has dramatically reduced cichlid numbers, particularly haplochromines (Witte et al., 2000). Witte et al. (1992) suggest that approximately 200 of the more than 300 endemic haplochromine species of the Mwanza Gulf, Lake Victoria, have already disappeared or are now threatened with extinction. Captive breeding of endangered cichlids is one means of trying to aid declining populations and efforts are underway (Reid, 1990; Fiumera et al., 1999, Silva et al., 2001). Insight into the hormonal pheromone systems of cichlids may provide potential means of assisting captive breeding efforts.

The objective of this research was to further characterize the hormonal pheromone system of *H. burtoni* by determining: 1) what steroids are detected by *H. burtoni*, 2) their ability to discriminate detected steroids, and 3) whether detectable steroids have specific biological functions. The results suggest that *H. burtoni* employ a complex hormonal pheromone system, and provide a basis for future studies.

LITERATURE CITED

Adams, M.E., Teeter, J.A., Katz, Y., and Johnsen, P.B. 1987. Sex pheromones of the sea lamprey, *Petromyzon marinus*: steroid studies. *J. Chem. Ecol.* **13**: 387-395.

Ali, S.A., Schoonen, W.G.E.J., Lambert, J.G.D., Van Den Hurk, R., and Van Oordt, P.G.W.J. 1987. The skin of the male African catfish, *Clarias gariepinus*: A source of steroid glucuronides. *Gen. Comp. Endocrinol.* **66**: 415-424.

Alimeida, O.G., Barata, E.N., Hubbard, P.C., and Canario, A.V.M. 2003. Urination rate of male tilapia (*Oreochromis mossambicus*) is highly dependent on social context. Abstract, Society for Experimental Biology Annual Main Meeting. March, 2002 (Southhampton, UK).

Allen, E.E., and Fernald, R.D. 1985. Spectral sensitivity of the African cichlid fish, *Haplochromis burtoni*. *J. Comp. Physiol. A.* **157**: 360-374.

Bjerselius, R., Li, W., Teeter, J.H., Seeyle, J.G., Johnsen, P.B., Maniak, P.J., Grant, G.C., Polkinghorne, C.N., and Sorensen, P.W. 2000. Direct behavioural evidence

that unique bile acids released by larval sea lamprey (*Petromyzon marinus*) function as a migratory pheromone. *Can. J. Fish. Aquatic. Sci.* **57**: 557-569.

Colombo, L., Marconato, A., Belvedere, P.C., and Frisco, C. 1980. Endocrinology of teleost reproduction: A testicular steroid pheromone in the black goby, *Gobius jozo* L. *Boll. Zool.* **47**: 355-364.

Crapon de Caprona, M.D. 1974. The effect of chemical stimuli from conspecifics on the behavior of *Haplochromis burtoni*. *Experientia.* **30**: 1394-1395.

Crapon de Caprona, M.D. 1980. Olfactory communication in a cichlid fish, *Haplochromis burtoni*. *Z. Tierpsychol.* **52**: 113-134.

Dulka, J.G., Stacey, N.E., Sorensen, P.W., and Van Der Kraak, G.J. 1987. A steroid sex pheromone synchronizes male-female spawning readiness in goldfish. *Nature.* **325**: 251-253.

Fernald, R.D. 1977. Quantitative behavioral observations of *Haplochromis burtoni* under semi-natural conditions. *Anim. Behav.* **25**: 643-653.

Fernald, R.D., and Hirata, N.R. 1977a. Field study of *Haplochromis burtoni*: quantitative behavioral observations. *Anim. Behav.* **25**: 964-975.

Fernald, R.D and Hirata, N.R. 1977b. Field study of *Haplochromis burtoni*: Habitats and co-habitant. *Environ. Biol. Fish.* **2**: 299-308.

Fernald, R.D. 1985a. Eye movements in the African cichlid fish, *Haplochromis burtoni*. *J. Comp. Physiol. A.* **156**: 199-208.

Fernald, R.D. 1985b. Growth of the teleost eye: Novel solutions to complex constraints. *Environ. Biol. Fish.* **13**: 113-123.

Fernald, R.D. 2002. Social regulation of the brain: status, sex, and size. pp 435-444. In *Hormones, Brain, and Behavior volume 2.* Edited by D.W. Pfaff, A.P. Arnold, A.M. Etgen, S.E. Fahrbach and R.T. Rubin. Academic Press, San Diego.

Fiumera, A.C., Wu, L., Parker, P.G., and Fuerst, P.A. 1999. Effective population size in the captive breeding program of the Lake Victoria cichlid *Paralabidochromis chilotes*. *Zoo. Biol.* **18**: 215-222.

Frade, P., Hubbard, P.C., Barata, E.N., and Canario, A.V.M. 2002. Olfactory sensitivity of the Mozambique tilapia to conspecific odours. *J. Fish. Biol.* **61**: 1239-1254.

Frade, P., Barata, E.N., and Canario, A.V.M. 2002. Olfactory detection of a putative reproductive pheromone in tilapia (*Oroechromis mossambicus*). Abstract, Society for Experimental Biology Annual Main Meeting. March, 2002 (Southhampton, UK).

Francis, R.C., Jacobson, B., Wingfield, J.C., and Fernald, R.D. 1992. Castration lowers aggression but not social dominance in male *Haplochromis burtoni*. *Ethology*. **90**: 247-255.

Goetz, F.W. 1983. Hormonal control of oocyte final maturation and ovulation in fishes. pp 117-170. In *Fish Physiology, volume IXB, Behavior and Fertility control*. Edited by W.S. Hoar, D.J. Randall, and E.M. Donaldson. Academic Press, New York.

Heiligenberg, W. 1973. Random processes describing the occurance of behavioral patterns in a cichlid fish. *Anim. Behav.* **21**: 169-182.

Heiligenberg, W. and Kramer, U. 1972. Aggressiveness as a function of external stimulation. *J. Comp. Physiol.* **77**: 332-340.

Hofmann, H.A., Benson, M.E., and Fernald, R.D. 1999. Social status regulates growth rate: Consequences for life history strategies. *Proc. Natl. Acad. Sci.* **96**: 14171-14176.

Kirchshofler, R. 1953. Aktionssystem des Maulbrüters *Haplochromis desfontainesii*. *Z. Tierpsychol.* **10**: 297-218.

Kroeger, R.H.H., and Fernald, R.D. 1994. Regulation of eye growth in the African cichlid fish, *Haplochromis burtoni*. *Vision Res.* **34**: 1807-1814.

Kroeger, R.H.H., Campbell, M.C.W., Munger, R., and Fernald, R.D. 1994. Refractive index distribution and spherical aberration in the crystalline lens of the African cichlid fish, *Haplochromis burtoni*. *Vision Res.* **34**: 1815-1822.

Li, W. 1994. The olfactory biology of adult sea lamprey, *Petromyzon marinus*. Ph.D. Dissertation, University of Minnesota, St. Paul.

Li, W., Sorensen, P.W., and Gallaher, D.D. 1995. The olfactory system of migratory adult sea lamprey (*Petromyzon marinus*) is specifically and acutely sensitive to unique bile acids released by conspecific larvae. *J. Gen. Physiol.* **105**: 569-587.

Li, W. and Sorensen, P.W. 1997. Highly independent olfactory receptor sites for

naturally occurring bile acids in the sea lamprey, *Petromyzon marinus*. *J. Comp. Physiol. A.* **180**: 429-438.

Li, W., Scott, A.P., Siefkes, M.J., Yan, H., Liu, Q., Yun, S., and Gage, D.A. 2002. Bile acid secreted by male sea lamprey that acts as sex pheromone. *Science*. **296**: 138-141.

Liley, N.R. 1982. Chemical communication in fish. *Can. J. Fish. Aquatic. Sci.* **39**: 22-35.

Mack, A.F. and Fernald, R.D. 1992. Control of vertebrate retinal cell production. *Exp. Neurol.* **115**: 65-68.

Matthiessen, P. and Logan. 1984. Low concentration effects of endosulfan insecticide on reproductive behaviour in the tropical cichlid fish *Saratherodon mossambicus*. *Bull. Environ. Contam. Toxicol.* **53**: 187-196.

Mayer, W.E., Tichy, H., and Klein, J. 1998. Phylogeny of African cichlid fishes as revealed by molecular markers. *Heredity*. **80**: 702-714.

Meyer, A., Kocher, T.D., Basasibwaki, P., and Wilson, A.C. 1990. Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. *Nature*. **347**: 550-553.

Meyer, A. 1993. Phylogenetic relationships and evolutionary processes in East African cichlid fishes. *Trends Ecol. Evol.* **8**: 279-284.

Mosler 1985. 1985. Making the decision to continue the fight or to flee: An analysis of contests between male *Haplochromis burtoni* (Pisces). *Behaviour*. **92**: 129-145.

Murphy, C.A., Maniak, P.J., and Sorensen, P.W. 2000. Functional and biochemical characterization of a novel sex pheromone in the Eurasian ruffe, *Gymnocephalus cernuus*. p 211. In *Proceedings of the 9th International Zebra Mussel and Aquatic Nuisance Species Conference*. Duluth, MN, April 16-30, 1999. The Professional Edge, Pembroke, Ontario.

Murphy, C.A., Stacey, N., and Corkum, L.D. 2001. Putative steroidal pheromones in the round goby, *Neogobius melanostomus*: olfactory and behavioral responses. *J. Chem. Ecol.* **27**: 443-470.

Nelson, J. S. 1994. Fishes of the World, 3rd. ed. John Wiley and Sons, New York.

Nelissen, M.H.J. 1977. Sound production by *Haplochromis burtoni* (Gunther) and

Tropheus moorii Boulenger (Pisces, Cichlidae). *Ann. Soc. R. Zool. Belg.* **106**: 155-166.

Nevitt, G.A. 1991. Do fish sniff? A new mechanism of olfactory sampling in pleuronectid flounders. *J. Exp. Biol.* **157**: 1-18.

Poling, K.R., Fraser, E.J., and Sorensen, P.W. 2001. The three steroidal components of the goldfish preovulatory pheromone signal evoke different behaviors in males. *Comp. Biochem. Physiol. B.* **129**: 645-651.

Reid, G.M. 1990. Captive breeding for the conservation of cichlid fishes. *J. Fish. Biol.* **37**: 157-166.

Resink, J.W., Schoonen, W.G.E.J., Albers, P.C.H., File, D.M., Notenboom, C.D., Van Den Hurk, R., and Van Oordt, P.G.W.J. 1989a. The chemical nature of sex attracting pheromones from the seminal vesicle of the African catfish *Clarias gariepinus*. *Aquaculture.* **83**: 137-151.

Resink, J.W., Voorthius, P.K., Van Den Hurk, R., Peters, R.C., and Van Oordt, P.G.W.J. 1989b. Steroid glucuronides of the seminal vesicle as olfactory stimuli in African catfish *Clarias gariepinus*. *Aquaculture.* **83**: 153-166.

Robison, R.R., Fernald, R.D., and Stacey, N.E. 1998. The olfactory system of a cichlid fish responds to steroidal compounds. *J. Fish. Biol.* **53**: 226-229.

Sakai, H., and Yoshii, K. 1990. A possibility of species discrimination by olfaction in the cyprinid fish genus (*Tribolodon*). *Jap. J. Ichthyol.* **37**: 194-197.

Salzburger, W., Meyer, A., Baric, S., Verheyen, E., and Sturmbauer, C. 2002. phylogeny of the Lake Tanganyika cichlid species flock and its relationship to the central and east African haplochromine cichlid fish faunas. *Syst. Biol.* **51**: 113-135.

Seehausen, O. 2002. Patterns of fish radiation are compatible with Pleistocene dessication of Lake Victoria and 14,600 year history for its cichlid species flock. *Proc. R. Soc. Lond. B. Biol. Sci.* **269**: 491-497.

Silva, E.I.L., Nathanael, S., Jayasinghe, R.P.P.K., and Amarasinghe, U.S. 2001. Some aspects of colonization success of three species of exotic cichlids in a hydropower reservoir (Victoria) in Sri Lanka. p 629. In *6th Asian Fisheries Forum Book of Abstracts.* Asian Fisheries Society 2001. Koahsiung, Taiwan.

Soma, K.K., Francis, R.C., Wingfield, J.C., and Fernald, R.D. 1996. Androgen regulation of hypothalamic neurons containing gonadotropin-releasing hormone in a cichlid fish: Integration with social cues. *Horm. Behav.* **30**: 216-226.

Sorensen, P.W. and Wyatt, J. 2000. Pheromones. pp 1193-1195. In *The Corsini Encyclopedia of Psychology and Behavioral Science, Third edition.* Edited by W.E. Craighead and C.B. Nemeroff. John Wiley and Sons, New York.

Sorensen, P.W., and Scott, A.P. 1994. The evolution of hormonal sex pheromones in teleost fish: Poor correlation between the pattern of steroid release by goldfish and olfactory sensitivity suggest that these cues evolved as a result of chemical spying rather than signal specialization. *Acta. Scand. Physiol.* **152**: 191-205.

Sorensen, P.W., Brash, A.R., Goetz, F.W., Kellner, R.G., Bowdin, L., and Vrieze, L.A. 1995. Origins and functions of F prostaglandins as hormones and pheromones in the goldfish. pp 244-248. In *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish.* Edited by F.W. Goetz and P. Thomas, P. FishSymp 95, University of Texas at Austin.

Sorensen, P.W. and Stacey, N.E. 1999. Evolution and specialization of fish hormonal pheromones. pp 15-47. In *Advances in Chemical Signals in Vertebrates.* Edited by R. E. Johnston, D. Muller-Schwarze, and P.W. Sorensen. Kluwer Academic, New York.

Stacey, N.E. 1976. Effects of indomethacin and prostaglandins on spawning behavior of female goldfish. *Prostaglandins.* **12**: 113-128.

Stacey, N.E 1987. Roles of hormones and pheromones in fish reproductive behavior. pp 28-69. In *Psychobiology of Reproduction: An Evolutionary Perspective.* Edited by D. Crews. Prentice Hall, New Jersey.

Stacey, N and P.W. Sorensen. 2002. Hormonal pheromones in fish. pp 375-434. In *Hormones, Brain, and Behavior volume 2.* Edited by D.W. Pfaff, A.P. Arnold, A.M. Etgen, S.E. Fahrbach and R.T. Rubin. Academic Press, San Diego.

Stacey, N.E., Sorensen, P.W., Van Der Kraak, G.J., and Dulka, J.G. 1989. Direct evidence that 17α,20β-dihydroxy-4-pregnen-3-one functions as a goldfish primer pheromone: Preovulatory release is closely associated with male endocrine responses. *Gen. Comp. Endocrinol.* **75**: 62-70.

Staeck, W. and Linke, H. 1996. Cichlids from Eastern Africa, 2nd ed. Tetra-Press, Germany.

Staissny, M.L. and Meyer, A. 1999. Cichlids of the rift lakes. *Sci. American.* **280**: 64-69.

Sturmbauer, C. and Meyer, A. 1993. Mitochondrial phylogeny of the endemic mouthbrooding lineages of cichlid fishes from Lake Tanganyika in eastern Africa. *Mol. Biol. Evol.* **10**: 751-768.

Tavolga, W.N. 1956. Visual, chemical, and sound stimuli as cues in the sex discriminatory behavior of the gobiid fish, *Bathygobius soporator*. *Zoologica.* **41**: 49-64.

Turner, G.F., Seehausen, O., Knight, M.E., Allender, C.J., and Robinson, R.L. 2001. How many species of cichlid fishes are there in African lakes? *Mol. Ecol.* **10**: 793-806.

White, S.A. and Fernald, R.D. 1993. Gonadotropin-releasing hormone-containing neurons change size with reproductive state in female *Haplochromis burtoni*. *J. Neurosci.* **13**: 434-441.

Witte, F., Goldschmidt, T., Wanink, J.H., van Oijen, M., Witte-Maas, E., and Bouton, N. 1992. The destruction of an endemic species flock: Quantitative data on the decline of the haplochromine cichlids of Lake Victoria. *Environ. Biol. Fishes.* **34**: 1-28.

Witte, F., Msuku, B.S., Wanink, J.H., Seehausen, O., Katunzi, E.F.B., Goudswaard, P.C., and Goldschmidt, T. 2000. Recovery of cichlid species in Lake Victoria: an examination of factors leading to differential extinction. *Rev. Fish. Biol. Fish.* **10**: 244-241.

Zheng, W. and Stacey, N.E. 1996. Two mechanisms for increasing milt volume in male goldfish. *J. Exp. Zool.* **276**: 287-295.

2. Olfactory Response to Steroid Hormones

2.1 INTRODUCTION

Hosoya and Yoshida (1937) first reported electrical activity in the olfactory epithelium of dogs, triggering interest in the capabilities of electrodes to capture olfactory responses. Ottoson (1956) followed up this work with studies on frog and rabbit olfaction and introduced the term electro-olfactogram (EOG), to describe a non-invasive extracellular recording technique that measures the negative electrical potential of the olfactory epithelium produced in response to odor-receptor binding. The EOG has since been used by numerous investigators to evaluate and characterize the response of cellular populations of olfactory receptor neurons. The majority of EOG studies have been performed on frog, salamander, rat, mouse, and dog preparations (see Scott and Scott-Johnson, 2002 for review), although a significant number of studies have focused on fish. The olfactory sense has been highly conserved (Sorensen and Caprio, 1997; Nakanishi, 1995), and insight into fish olfaction may help us understand olfaction in higher vertebrates.

Water-borne steroids, prostaglandins, and their metabolites are known to influence both reproductive physiology and/or behavior in a wide variety of fish species (Sorensen and Stacey, 1999; Stacey and Sorensen, 2002). These hormonal pheromones have been shown to induce ovulation in zebrafish, *Danio rerio* (Van Den Hurk et al., 1987), spermiation in goldfish, *Carassius auratus* (Dulka et al., 1987), and ventilation in the round goby, *Neogobius melanostomus* (Murphy et al., 2001), and to act as female attractants in the black goby *Gobius jozo* = *G. niger* (Colombo et al., 1980) and guppy *Poecilia reticulata* (Johansen, 1985). Underwater EOG recordings have accelerated our understanding of the nature of hormonal pheromones through extensive characterization of the olfactory organ. More recently, EOG studies have been used to examine transduction processes associated with olfactory response in mice (Brunet et al., 1996; Wong et al., 2000). EOG studies can be used to estimate a pheromone's active space: i.e. the maximum volume in which it can be detected (Sorensen et al., 2000). EOG recordings can also determine the olfactory

sensitivity and specificity of a species to an assortment of hormones and hormone metabolites. Further cross-adaptation and binary mixture studies (e.g. Li and Sorensen, 1997) can evaluate the ability of olfactory receptor neurons to discriminate between detected odors.

In cross-adaptation, the olfactory epithelium is first exposed to one detected steroid (test steroid). The olfactory epithelium is given time to recover from the first exposure and then is chronically exposed to a second detected steroid (adapting steroid) until adaptation has occurred (typically less than one minute). During continued application of the adapting solution, the olfactory epithelium is re-exposed to the test steroid. If the EOG response to the test steroid is unaffected by adaptation, the two steroids are assumed to act via different receptor mechanisms and are potentially discriminated. If the EOG response to the test solution is reduced or abolished due to adaptation, the two steroids are assumed to act via the same receptor mechanism and may not be discriminated. The significance of cross-adaptation is that two steroids acting through the same receptor mechanism are likely to have the same biological function (Caprio and Byrd, 1984; Li and Sorenson, 1999).

Another means of evaluating the ability to discriminate detected odors is through binary mixture tests that compare the response of odor mixtures to those of individual components. Additivity shown by mixtures can be attributed to the stimulation of multiple receptor mechanisms because odors are tested at concentrations near the peak of their dose-response curves (Li and Sorensen, 1997; Kang and Caprio, 1991; Caprio et al., 1989). Under natural conditions, fish are exposed to a complex mixture of odors, and it is important to understand the role that mixtures play at the sensory level to better understand olfactory processes as a whole.

EOG tests were used in this study to examine olfactory sensitivity and responsiveness of *Haplochromis burtoni* to a variety of steroid and prostaglandin hormones. I attempted to characterize olfaction in *H. burtoni* by determining what steroids and prostaglandins were detected, threshold concentrations necessary to elicit responses, and the ability of *H. burtoni* to discriminate detected compounds.

The round goby (*Neogobius melanostomus*) has recently been shown to increase ventilation rate in response to steroid hormone exposure (Murphy et al., 2001). Round gobies detect a variety of 18, 19, and 21-carbon steroids through four classes of olfactory receptor mechanisms. Exposure to steroids from three of these classes (estrone, 17β-estradiol-3β-glucuronide, and etiocholanolone) increases ventilation rate in males, whereas only etiocholanolone stimulates ventilation rates in females. These ventilation increases may facilitate odor detection by increasing water flow across the olfactory epithelium (e.g. Nevitt, 1991). The sexually dimorphic behavioral responses shown by *N. melanostomus* appear to be androgen-dependent, as females implanted with methyl-testosterone exhibit male-typical ventilatory response after 8-10 days (Murphy and Stacey, 2002). This androgen-dependent response to estrogens may facilitate male mate choice by providing a means by which males can assess female quality, discriminate between males and females, or discriminate between preovulatory and ovulated females (Murphy and Stacey, 2002).

Extensive work on the sea lamprey, *Petromyzon marinus,* suggests the use of both bile acids and sex steroids as pheromones (Li and Sorensen, 1997; Adams et al., 1987). EOG studies have shown that several sulfated sex steroids stimulate olfactory response of sea lampreys (Li, 1994). Selection of spawning streams by migrating adults is mediated by larval bile steroids (Li et al., 1995; Bjerselius et al., 2000). Water conditioned by spermiating male lampreys, but not prespermiating males or females, influences female locomotor activity and search behavior in females, even when placed up to 65 m downstream of the odor source (Li et al., 2002). These results suggest that a pheromone released by male lampreys in spawning streams acts as an attractant signal for females that has a large active space. Specialized glandular gill cells found only in spermiating males (Li et al., 2002) may facilitate this signaling and provide a means by which lampreys can communicate over great distances.

The hormonal pheromone system of goldfish (*Carassius auratus*) is best understood. Goldfish are a non-pairbonding species in which multiple males engage in intense sperm competition. A steroid hormone, 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P), is produced in female goldfish follicles prior to ovulation. 17,20β-P induces final oocyte maturation (Goetz, 1983). 17,20β-P, its metabolite 17,20β-P-

sulfate, and androstenedione are released into the water where they function as a preovulatory pheromone, elevating blood gonadotropin-II (GtH-II) concentration and milt volume in males (Dulka et al., 1987, Stacey et al., 1989). Blood plasma concentrations of these steroid hormones vary with female reproductive status, with the ratio of 17,20β-P to androstenedione increasing as the female preovulatory GtH-II surge progresses (Sorensen and Scott, 1994). Recent evidence suggests that the three components of the preovulatory pheromone elicit different sets of behaviors. Androstenedione appears to stimulate aggressive behavior (pushing) in males whereas 17,20β-P stimulates low levels of persistent courtship (nudging and chasing). 17,20β-P-sulfate triggers more pronounced transient courtship behaviors (nudging and chasing) (Poling et al., 2001).

At ovulation, the movement of mature oocytes into the oviduct stimulates follicular synthesis of prostaglandin F2α (PGF2α) (Sorensen et al., 1995). PGF2α acts hormonally to trigger female spawning behavior (Stacey, 1976) and, together with a metabolite (15-keto-PGF2α), is released to the water to function as a postovulatory pheromone stimulating courtship behavior and elevating blood GtH-II concentration and milt volume in males (Zheng and Stacey, 1996).

Other than early work with the black goby (*Gobius jozo = G. niger*) (Colombo et al., 1980), recent studies on the round goby (Murphy et al., 2001), and evident use of alarm and preovulatory sex pheromones in the Eurasian ruffe (*Gymnocephalus cernuus*) (Murphy et al., 2000), there is no evidence of hormonal pheromones in the order Perciformes. Perciformes is an ecologically important group of marine and freshwater fishes (Nelson, 1994) that contain cichlids, the second most abundant family of fish. In the goldfish system, the biological functions of specific detectable hormones are well understood. Much less is known about the hormonal pheromone systems of pair bonding species, such as *Haplochromis burtoni*, an African cichlid. Cichlids are exceptional candidates for pheromone study because they have mating systems that are quite different from those in species where hormonal pheromones have been studied, enabling interesting comparisons with known species. Cichlids are also very diverse (Staeck and Linke, 1996) making comparative studies among

controlled solenoid switched between this background water and 2-second pulses of test solutions. Signals elicited from the olfactory epithelium were amplified (Grass P-18 DC amplifier) and digitized (National Instruments Lab-PC converter), and responses were recorded for 10 seconds after exposure. Fish were anesthetized for up to four hours and, after testing was completed, the gills were perfused with background water until the fish revived and could be returned to an aquarium.

After an anesthetized fish was secured in the water bath and electrodes were positioned, recording began by determining olfactory response to 10^{-5} M L-alanine. If response to alanine was adequate (> 2 mV), recording continued. If alanine response was negligible, I repositioned the polyethylene tube delivering odor pulses and/or the recording electrode until a stable recording was obtained. EOG responses were quantified by measuring peak phasic voltage change from baseline during odor exposure. Response to a background solution of 0.015% ethanol (concentration of ethanol solvent in steroid stock solutions; typically < 0.3 mV) was subtracted from the response magnitudes of all subsequently tested compounds. During the course of a recording, response to 10^{-5} M L-alanine was closely monitored to ensure that olfactory sensitivity and recording conditions remained consistent. To minimize variation in recording conditions during a recording session, magnitudes of responses were expressed as a percentage of that elicited by the most recent response to the alanine standard, assuming the olfactory epithelium maintains a constant ratio of responsiveness to L-alanine and test odors.

2.3.2 Tested Odors

The olfactory epithelium of *H. burtoni* was exposed to more than 160 steroids and eight prostaglandins (Appendix 1) at 10^{-9} M concentrations to determine which compounds induced consistent olfactory response. All odors tested were obtained through Sigma (St. Louis, MO), Steraloids (Wilton, NH), Cayman Chemical Company (Ann Arbor, MI), or as gifts from Dr. A.P. Scott (Lowestoft, UK). The L-alanine standard was prepared as a 10^{-2} M stock solution in double-distilled, de-ionized water, and test solutions (10^{-5} M) were made as desired through dilution in background water. Steroids were first prepared as 10^{-3} M stock solutions in 95%

ethanol (unconjugated and sulfated steroids) or 50:50 ethanol-water (glucuronated steroids). Working solutions (10^{-6} M) were prepared in triplicate by diluting stock solutions in double-distilled, de-ionized water and stored in 20 ml glass scintillation vials at 4^0 C. Test solutions were prepared as desired immediately before recording through serial dilution.

Six cichlids (4 males, 2 females) were initially exposed to 10^{-9} M test solutions of all compounds listed (Appendix 1). Fish were initially tested using mixtures of related compounds (4-10 compounds per mixture) to allow rapid screening of all steroids and prostaglandins. If a mixture elicited an EOG response, all individual components were then tested to determine which compound(s) was detected. Of the seventeen steroids that elicited EOG response (Table 2.1), eleven were chosen for this study based upon previous research (Robison et al. 1998), consistency of responsiveness, and preliminary cross-adaptation and binary mixture tests (data not shown). These steroids (hereafter *detected steroids*) include three 18-carbon, four 19-carbon, and four 21-carbon steroids.

2.3.3 Concentration-response studies

To determine and compare olfactory sensitivity to detected steroids, concentration-response profiles were generated for all eleven steroids. Concentration-response tests began at 10^{-12} M and increased by log molar increments to 10^{-8} M with approximately 1 minute between exposures. Tests for each steroid were performed on six females and between 5 and 11 males. Male and female response magnitudes to each steroid at 10^{-8} M were compared using an unpaired t-test (or Mann-Whitney U test) with Bonferonni correction. EOG sensitivity (threshold) was determined by examining response magnitudes of all fish used in concentration response studies. For each steroid, threshold was determined by the lowest dose that elicited a response in the majority of fish tested.

2.3.4 Cross-adaptation studies

EOG cross-adaptation was used to determine whether *H. burtoni* can discriminate the detected steroids at the sensory level by establishing the number of receptor

mechanisms that are present. A *receptor mechanism*, as used in this thesis, is the physiological entity responsible for an EOG response; it includes the receptor cell and associated transduction pathway(s) (Sorenson et al., 1995a). Cross-adaptation began by first determining olfactory response to all eleven detected steroids at 10^{-8} M. One of the detected steroids was then chosen as the adapting steroid and was prepared as 10^{-8} M adapting solution by diluting 2 ml of 10^{-5} M steroid solution into 2 L of background water. A portion of this adapting solution was used to perfuse the olfactory epithelium during chronic exposure to the adapting compound. Test solutions were then administered at 10^{-8} M (prepared by diluting 40 µl of 10^{-5} M steroid solution into 40 ml of adapting solution) in the same sequence used during the pretest. The first steroid tested in this manner after chronic exposure was always the adapting steroid itself (self-adapting control). In a typical cross-adaptation test, all ten other steroids in adapting steroid solution were then tested sequentially interspersed with exposures to alanine in adapting steroid solution. All eleven steroids were used as adapting stimuli.

Although cross-adaptation tests assume that a brief (2 second) exposure to a steroid will not influence responsiveness to that same odor when presented a short time later, Murphy et al. (2001) showed that in the round goby, olfactory response to some steroids is reduced by brief exposure. To determine whether this is also the case in *H. burtoni*, a series of sequential exposure tests were performed in the same manner as a typical cross-adaptation, with the exception that fish were not chronically exposed to an adapting steroid. These tests evaluated the impact of sequential steroid exposure on EOG response magnitude.

There are several procedures for analyzing cross-adaptation data (see Li and Sorenson, 1997; Murphy et al., 2001). I chose to use the most conservative means possible to avoid the potential influence of repeated steroid exposure on EOG response reduction. Percent initial response (%IR) was determined by comparing response during adaptation to response given during the pretest. For each test steroid, %IR was then compared to %IR of the adapting steroid, using Kruskal-Wallis nonparametric ANOVA followed by Dunnett's tests. If response to a test steroid during adaptation was significantly greater than to the self-adapting control, the two

steroids were considered to act via different receptor mechanisms. This means of analyzing cross-adaptation results is conservative in that in order to conclude that two odors act via the same receptor mechanism, the adaptation response of the test solution must mimic that of the self-adapted control. This analysis controlled for any false-positives (reductions in response magnitude during adaptation) that may be attributable to sequential steroid exposure (e.g. Murphy et al., 2001) and/or chronic exposure to a steroid in general.

2.3.5 Binary mixture studies

In binary mixture studies, EOG responses to two steroids (A and B) were first measured at 10^{-8} M (this concentration was chosen to best provide equipotentcy while minimizing desensitization). The responses recorded were denoted RA and RB. EOG responses were then measured at twice the initial concentration and resulting response were denoted R2A and R2B. Finally, EOG response to a mixture of compounds A and B at 10^{-8} M (initial concentration) was measured and this response denoted as RAB. Approximately 90 seconds were allowed between all exposures. Binary mixture experiments were used to generate two indices, the independent component index (ICI) and the mixture discrimination index (MDI). Relative EOG responses were used to calculate the ICI and MDI of each mixture combination using the following formulae (Li and Sorenson, 1997; Kang and Caprio 1991):

$$ICI = RAB/(RA + RB)$$

$$MDI = RAB/(0.5* (R2A + R2B))$$

These two indices use different means to evaluate whether the components of a binary mixture act via the same receptor mechanism. The ICI compares the response to the mixture to the sum of the responses generated from each of the independent components. This approach assumes that when two independently acting odors are tested as a mixture the response will be perfectly additive. Thus, the ICI should be 1 when two odors act via different receptor mechanisms. ICI values that are less than 1 suggest a lack of independence.

The MDI characterizes the response to the mixture when compared to the responses measured at twice the initial concentration (R2A and R2B). The MDI

should be 1 if the mixture components act via the same receptor mechanism. An MDI value greater than 1 is expected when the two components of a mixture show independence (additivity). This approach assumes that there will not be a difference in response magnitude between the initial exposures (RA and RB) and exposures to steroids tested at twice the initial concentrations (R2A and R2B). Thus, any additive effect seen during the mixture response can be attributed to the components acting independently and not to an increase in overall concentration. However, differences in concentration-response profiles can cloud the relationship between the MDI and receptor mechanism independence (Li and Sorenson, 1997).

EOG responses to nearly all of the possible binary combinations of the eleven detected steroids were tested. Several combinations including 5ß-pregnan-3α,17,21-triol-11,20-dione-3-glucuronide were omitted as it was clear through preliminary cross-adaptation and binary mixture tests that 5ß-pregnan-3α,17,21-triol-11,20-dione-3-glucuronide acts via the same receptor mechanism as 5ß-pregnan-3α,17-diol-20-one-glucuronide.

Male and female data (N = 6-9 for each sex) were combined for analysis. Individual ICI and MDI values were collected and grouped according to whether the components of the mixtures were detected via the same or different receptor mechanisms based on cross-adaptation studies. These two groups (within-group and across-group) were then compared using a Welch corrected t-test.

There was some desensitization seen during binary mixture testing which was attributed to the binary mixture procedure itself. The repeated steroid exposures of increasing concentration during binary mixture testing caused reduction in response magnitude to some odors as tests progressed. To correct for this effect, the level of response desensitization shown by each steroid during binary mixture testing was evaluated. Fish were exposed to a steroid at 10^{-8} M (first exposure), then at 2 x 10^{-8} M (second exposure), and then again at 10^{-8} M (third exposure). As in binary mixture tests, 90 seconds were allowed between exposures. The ratio of the third exposure to the first exposure (desensitization factor) was calculated for each steroid and analyzed using a Wilcoxon matched-pairs test. These values were then used to determine the level of additivity expected to occur during binary mixture testing. Since individual

steroid response is reduced because of the testing procedure, complete additivity of independently acting components is unlikely. The desensitization factor for each steroid was multiplied by its respective initial response at 10^{-8} M to generate new RA and RB measurements denoted RA' and RB'. Therefore, the following modification to the accepted ICI formula was made:

$$ICI' = RAB/(RA' + RB')$$

2.4 RESULTS

2.4.1 EOG response of *H. burtoni*

Responses to background water and to a 0.015% ethanol control were negligible (typically < 0.3 mV). When minor surgery was performed to remove some skin surrounding the naris, EOG responsiveness was usually reduced for approximately 20 minutes until it returned to a more consistent level. With the exception of a few (< 5) cases, fish always recovered after EOG testing which lasted from 1 to 4 hours per fish.

2.4.2 Tested odors

Based on the EOG recordings, the olfactory epithelium of *H. burtoni* detects (typical threshold 10^{-10} M) 17 of the conjugated steroids tested but not prostaglandins or unconjugated steroids. Of these 17 conjugated steroids (Table 2.1), six (three 18-carbon, two 19-carbon, and one 21-carbon steroids) were excluded from further study and eleven were chosen based upon previous research (Robison et al., 1998), consistency of responsiveness, and preliminary cross adaptation and binary mixture tests. The 11 steroids chosen for further study were from 3 major steroid classes: C18 steroids (17ß-estradiol-17-glucuronide, 17ß-estradiol-3-sulfate, and 17ß-estradiol-disulfate); C19 steroids (epiandrosterone-sulfate, etiocholanolone-glucuronide, testosterone-sulfate, and dehydroepiandrosterone-sulfate; DHEA-s); and C21 steroids (5α-pregnan-3ß-ol-20-one-sulfate, 5ß-pregnan-3α,17-diol-20-one-glucuronide, 5ß-pregnan-3α,17,21-triol-11,20-dione-3-glucuronide, and pregnenolone-sulfate).

There is little evidence of sexual dimorphism in EOG response at 10^{-8} M (Figure 2.1). Males had greater absolute response magnitudes in general and these differences were significant for three steroids (etiocholanolone-glucuronide, 17ß-estradiol-3-sulfate, and pregnenolone-sulfate). However, when expressed as relative response (percentage of the response elicited by the most recent alanine standard), only the responsiveness to 17ß-estradiol-3-sulfate was sexually dimorphic, being larger in males than in females.

2.4.3 Concentration-response studies

Olfactory response magnitude and sensitivity to detected steroids was determined through concentration-response studies (Figures 2.2a, b). Male and female concentration-response curves were similar, with typical thresholds of 10^{-10} M. Males were most sensitive to etiocholanolone-glucuronide, which elicited a response at 10^{-11} M. Males were slightly more sensitive to etiocholanolone-glucuronide, 17ß-estradiol-3-sulfate, and 5α-pregnan-3ß-ol-20-one-sulfate than females. Threshold EOG response to epiandrosterone-sulfate, 17ß-estradiol-17-glucuronide, 5α-pregnan-3ß-ol-20-one-sulfate, and pregnenolone-sulfate was 10^{-9} M. For females, threshold for 5α-pregnan-3ß-ol-20-one-sulfate was reached at 10^{-8} M. Response plateau was not reached for any steroid except 5ß-pregnan-3α,17-diol-20-one-glucuronide, and there was a sharp increase in response magnitude for all steroids between 10^{-9} and 10^{-8} M. Response magnitude to all steroids continued to increase through the upper concentration limit of these concentration-response studies (10^{-8} M).

2.4.4 Cross-adaptation studies

Cross-adaptation experiments suggest that the 11 steroids examined in this study are detected by 5 different receptor mechanisms (Figure 2.3a). These 5 receptor mechanisms can be described by the position and type of conjugate in the ligand: 3-sulfate, 17-sulfate, 3-glucuronide, 17-glucuronide, and 3,17-disulfate (Figure 2.3b).

The 3-sulfate group consists of epiandrosterone-sulfate, dehydroepiandrosterone-sulfate (DHEA-sulfate), 17ß-estradiol-3-sulfate, 5α-pregnan-3ß-ol-20-one-sulfate, and pregnenolone-sulfate (Table 2.2). There is very good reciprocity in the ability of

each of these steroids to significantly reduce responsiveness during adaptation (Figure 2.3a). As adapting solutions, epiandrosterone-sulfate, DHEA-sulfate, and 5α-pregnan-3ß-ol-20-one-sulfate reduced response magnitudes to each of the other 3-sulfate steroids such that they were not significantly different from response to self-adapting control ($P > 0.05$). During adaptation to epiandrosterone-sulfate, DHEA-sulfate, or 5α-pregnan-3ß-ol-20-one-sulfate, responses to all other steroids were significantly greater than the self-adapting control ($P < 0.01$). EOG responses to all 3-sulfate steroids (except 5α-pregnan-3ß-ol-20-one-sulfate) were no different than the self-adapting control when 17ß-estradiol-3-sulfate was the adapting solution ($P > 0.05$). Response to 5α-pregnan-3ß-ol-20-one-sulfate was reduced but was still significantly greater than response to the self-adapting control ($P < 0.05$). Pregnenolone-sulfate did reduce the response magnitudes of other 3-sulfate steroids but only 5α-pregnan-3ß-ol-20-one-sulfate showed an adaptation response that was no different than the self-adapting control ($P > 0.05$).

As an adapting compound, testosterone-sulfate produced some ambiguous results. Although it clearly did not interact with any of the glucuronated steroids ($P < 0.01$), adaptation responses to all other steroids were reduced somewhat by chronic exposure to testosterone-sulfate. However, all adaptation responses were still significantly greater than response to the self-adapting control ($P < 0.05$).

The 3-glucuronide group consists of etiocholanolone-glucuronide, 5ß-pregnan-3α,17-diol-20-one-glucuronide, and 5ß-pregnan-3α,17,21-triol-11,20-dione-3-glucuronide (Table 2.2). This group exhibited clear interaction at the receptor level, as responsiveness during chronic exposure to each steroid was no greater than the respective self-adapting control ($P > 0.05$).

17ß-estradiol-17-glucuronide appears to act via its own receptor mechanism. During adaptation to 17ß-estradiol-17-glucuronide, response magnitudes of all steroids were significantly greater than the self-adapting control ($P < 0.01$).

17ß-estradiol-disulfate also appears to act via its own receptor mechanism. Response magnitudes of all steroids were significantly greater than the self-adapting control when 17ß-estradiol-disulfate was the adapting solution ($P < 0.01$).

EOG response magnitudes to all steroids were not significantly affected by prior exposure as seen in sequential exposure tests ($P > 0.05$) (Figure 2.3a).

2.4.5 Binary mixture studies

The repeated exposure to increasing concentrations of steroids in binary mixture tests has a desensitizing effect on *H. burtoni* EOG response (Figure 2.4). For this reason, binary mixes of unrelated odors would not be expected to induce completely additive EOG responses. Therefore, desensitization factors (see pages 28-29) were used to modify calculation of the ICI index. Although desensitization experiments yielded only two significant reductions in response (epiandrosterone-sulfate and 17ß-estradiol-17-glucuronide), the desensitization factor for each steroid was used to transform the data to adjust for the level of additivity expected in binary mixture tests. EOG response desensitization differed among tested steroids and so individual desensitization factors were calculated.

Results from binary mixture tests correlate well with cross-adaptation results, suggesting that the receptor mechanism groupings derived from cross-adaptation are valid. When ICI' values are combined into groups identified by cross-adaptation to represent the same receptor mechanism (within-group mixtures), the average value is 0.854, whereas the average ICI' value for across-group mixtures is 0.964; these values are significantly different from each other ($P < 0.05$) (Figure 2.5). Average MDI value for within-group mixtures (1.26) was significantly different than the average MDI value for across-group mixtures (1.45) ($P < 0.01$).

2.5 DISCUSSION

Male and female *H. burtoni* exhibit equal response magnitudes to detected steroids at 10^{-8} M, as seen in many other fishes including the round goby (Murphy et al., 2001), the sea lamprey *Petromyzon marinus* (Li and Sorenson, 1997), roach *Rutilus rutilus* (Lower et al., in press), *Puntius schwanenfeldi* (Cardwell et al., 1995), and goldfish (Sorenson et al., 1995b). The olfactory epithelium of *H. burtoni* is sensitive only to conjugated C18, C19, and C21 steroids but not prostaglandins. These steroids

appear to act through 5 independent receptor mechanisms that appear to respond to conjugate type and position. This suggests that type and position of conjugate present is of central importance in the discrimination of detected odors. Slight shifts in the carbon ring arrangement (e.g. switching from an α hydroxyl to a β hydroxyl on C17) can significantly affect EOG response to an odor. For example, whereas pregnenolone-sulfate appears to act through the 3-sulfate receptor mechanism, slight modifications in stereochemistry render closely related steroid metabolites undetectable. Thus, pregnenolone-glucuronide and an isomer of pregnenolone-sulfate containing an alcohol group on C17 are both undetectable (at 10^{-9} M) by *H. burtoni*. It is this remarkable specificity of receptors along with the enormous variety of potential steroid metabolites that lead to speculation that hormonal pheromones may provide a chemically-mediated isolating mechanism for sympatric species. Further, the complex mixes and blends of released hormones (in varying ratios depending on reproductive status) are expected to influence the function of these compounds in the receiver (Poling et al., 2001). Interestingly, and in marked contrast to the present findings, EOG studies of another cichlid, the Mozambique tilapia (*Oreochromis mossambicus*), failed to induce olfactory response to any of a small number of steroids including testosterone-sulfate (Frade et al., 2002).

Cross-adaptation studies are essential when using EOG recordings to characterize the olfactory organ because compounds detected through separate receptor mechanisms may have separate biological functions. The best understood example of this is in goldfish, where the three components of the preovulatory pheromone (17,20β-P, 17-20β-P-sulfate, and androstenedione) are detected through separate receptor mechanisms and where each has distinct effects on circulating gonadotropin-II (GtH-II) and behavior (Sorensen et al., 1995b; Poling et al., 2001).

EOG cross-adaptation studies in *H. burtoni* were successful in that there was nearly complete reciprocity of adaptation. Steroids either inhibited responses to each other when used as adapting stimuli or had no effect. For example, adaptation to etiocholanolone-glucuronide caused EOG response to 5β-pregnan-3α,17-diol-20-one-glucuronide to mimic that of the self-adapting control, and adaptation to 5β-pregnan-3α,17-diol-20-one-glucuronide caused EOG response to etiocholanolone-glucuronide

to mimic that of the self-adapting control as well. However, two members of the 3-sulfate group did not display this reciprocity. Adaptation to pregnenolone-sulfate did reduce EOG responsiveness to all other 3-sulfate steroids; only response to 5α-pregnan-3β-ol-20-one-sulfate was reduced to the extent that it was not significantly different than the self-adapting control. Similarly, adaptation to 17β-estradiol-3-sulfate caused significant reduction of EOG response to all other 3-sulfate steroids, except 5α-pregnan-3β-ol-20-one-sulfate. Despite these minor inconsistencies, I am confident in the receptor mechanism groups suggested by cross-adaptation studies as trends of reciprocity are clearly demonstrated.

Interpretation of cross-adaptation results in general can be problematic. Caprio and Byrd (1984) suggest that assigning detected compounds to receptor mechanism groups is difficult because of the possibility that olfactory receptor cells have multiple binding. The binding of an adapting steroid at one site may affect the binding properties of the other site(s). This may prevent reciprocal adaptation among similar compounds believed to share a receptor mechanism. Further, recent studies in the round goby show that brief (2 sec) steroid exposure can reduce response to that same odor when delivered 30 min later. This phenomenon can influence cross-adaptation results as EOG responsiveness during adaptation could be compromised regardless of the receptor mechanism(s) associated with the odors involved. However, this phenomenon was not observed in my cross-adaptation studies, as control adaptation tests revealed that EOG response to steroids was not affected by prior exposure.

Binary mixture experiments support the receptor mechanism groups established in cross-adaptation studies. There is a significant level of independence shown by across-group odor mixtures (a mixture containing two odors thought to act via different receptor mechanisms). The ICI' of across-group mixtures was 0.964 and the MDI of across-group mixtures was 1.45, values similar to those seen in other binary mixture studies (Caprio et al., 1989; Li and Sorenson, 1997).

Binary mixture results can be misleading because differences in concentration-response profiles of tested odors can cloud the relationship between the generated indexes and receptor mechanism independence. For example, two steroids that act through separate receptor mechanisms should show additivity when presented

together, as the mixture stimulates two independent receptor mechanisms. This should result in a mixture response equal to the sum of each component (ICI = 1). Ideally, these steroids are tested at concentrations that minimize any response magnitude increase caused by doubling the initial concentration (e.g. RA = R2A). Two steroids acting via the same receptor mechanism should therefore induce a mixture response that mimics that of one component tested individually at twice its initial concentration, or that of the average of the responses elicited by exposure to the two components at twice their initial concentrations, respectively. It is therefore critical to use equipotent odor concentrations in binary mixture tests and also to ensure that these concentrations represent the peak or plateau of their respective concentration-response curves. Further, because binary mix tests include the delivery of odors at twice the initial concentration, EOG response to odors in binary mix tests can be affected by previous odor exposure. Although previous odor exposure did not affect EOG response in cross-adaptation tests, there was a slight EOG response desensitization seen in binary mix tests. Because of these factors, a modified ICI calculation was developed (denoted ICI') to estimate the level of additivity expected in binary mixtures. The new independent component index (ICI') accounts for the response desensitization caused by the binary mixture test procedure. This response desensitization may be common, and could possibly explain less-than-perfect specificity of receptor sites (e.g. Caprio et al., 1989; Kang and Caprio, 1991) and mixture suppression (Daniel and Derby, 1991; Olson and Derby, 1995) seen in other studies.

EOG response profiles for tested steroids can also vary, with peak response amplitudes occurring at different times. These differences can have significant effects on binary mixture analysis and cloud the relationship between detected odors. Binary mixture analysis calculations assume that the response to a mixture will be equal to the sum of the responses generated by each individual component. However, if the two components do not induce peak response amplitude at the same time, this criterion cannot be met (see Appendix 2).

2.2 FISH

H. burtoni obtained from the Psychology department, Stanford University, and Biotope Imports, Montreal PQ, were used to establish a breeding colony at the University of Alberta. Fish were maintained in mixed-sex groups in aerated 300 liter fiberglass aquaria provided with flow-through dechlorinated water at 24-25^0 C under a constant photoperiod (14L:10D). Aquaria included gravel, artificial plant cover, rocks, and terra cotta plant pots to provide shelter. Fish were fed *ad libitum* with a variety of foods including Nutrafin flakes (Hagen), frozen brine shrimp (Hagen), and frozen bloodworms (Hagen).

Fish were sexed based upon size, coloration, and appearance of distinct egg spots on their anal fins. Males are larger and have either a light metallic blue or yellow basic body color, and may have bright red-orange humoral scales (Fernald, 1977). Females have a uniformly sandy-gray appearance with no blue or yellow body color and no red-orange humoral scales. Most importantly, males have 5 to 9 egg-spots on their anal fins, which are distinct because of a discrete orange border around each spot. Females may have similar markings on their anal fins, but these spots are always faint and are never made conspicuous by an orange border. Non-territorial males may assume a sandy-gray body color similar to that of females, but can still be distinguished from females based upon the appearance of anal fin spots.

Further, three other markings can be used to help differentiate between males and females. Adult male *H. burtoni* may display a lachrymal stripe (black bar running from eye to mouth), one or more eye-bars (black bars on the forehead), and an opercular spot. Females will generally not show any of these characteristics but may assume a lachrymal stripe and/or eye bar(s) when brooding or defending young. Because the lachrymal stripe can be turned on and off within seconds, and the eye-bars and opercular spots can be turned on and off within minutes, anal fin spots, basic body color, and humoral scale patch color should be used as the primary means of external sex differentiation.

Fish were bred at the University of Alberta by removing brooding females (with eggs or young in their mouths) from mixed-sex aquaria. Brooding females were placed individually into 80-100 L aquaria and removed approximately one week after release of their young. Young were fed powdered flake food (Hagen) and crushed bloodworms (Hagen) after approximately 3 weeks. These young were often mixed with young from other females and used to establish new mixed-sex stock aquaria. Adults bred at the University of Alberta, obtained directly from the Psychology department at Stanford University, and purchased from Biotope Imports, Montreal PQ were used in these experiments. All experiments performed on fish complied with Canadian Council on Animal Care guidelines.

2.3 METHODS

2.3.1 Electro-olfactogram (EOG) recording

Adult male (8-25 g) and female (5-20 g) fish were tested for olfactory sensitivity to approximately 170 steroids and prostaglandins using methods similar to those of Cardwell et al. (1995). Fish were anesthetized by immersion in 0.035% tricaine-methane-sulphonate (TMS; Syndel), wrapped in wet paper towel and secured in a temperature-controlled running water bath at 27^0 C. Fish were immersed on their side with just the dorsal head (eye and naris) out of the water. Wet paper towel was placed over the exposed eye. A polyethylene mouth tube delivered 0.02% TMS throughout the entire procedure. A glass capillary tube (75-150 μm tip diameter) filled with gelatin (8% in 0.6% NaCl) bridged the olfactory epithelium to Ag/AgCl electrodes filled with 3 M KCl. A reference electrode was placed in contact with the holding water while the recording electrode was placed over the olfactory epithelium. Often a small amount of skin surrounding the naris was removed to expose the olfactory lamellae and provide a better view for electrode placement. EOG response was typically reduced for a short time because of these naris cuts. Fish were allowed approximately 20 minutes after this procedure before testing began.

A polyethylene tube carrying dechlorinated tap water (hereafter *background water*) constantly perfused the olfactory epithelium and a custom built computer-

Figure 2.1. Comparison of male and female EOG response magnitudes (mean + S.E.M) to detected steroids (s = sulfate, g = glucuronide) (10^{-8} M) and an amino acid standard (10^{-5} M L-alanine). Sample sizes are indicated beside bars. Unpaired t-test (or Mann-Whitney U test) with Bonferonni correction. * $P < 0.01$ ** $P < 0.001$.

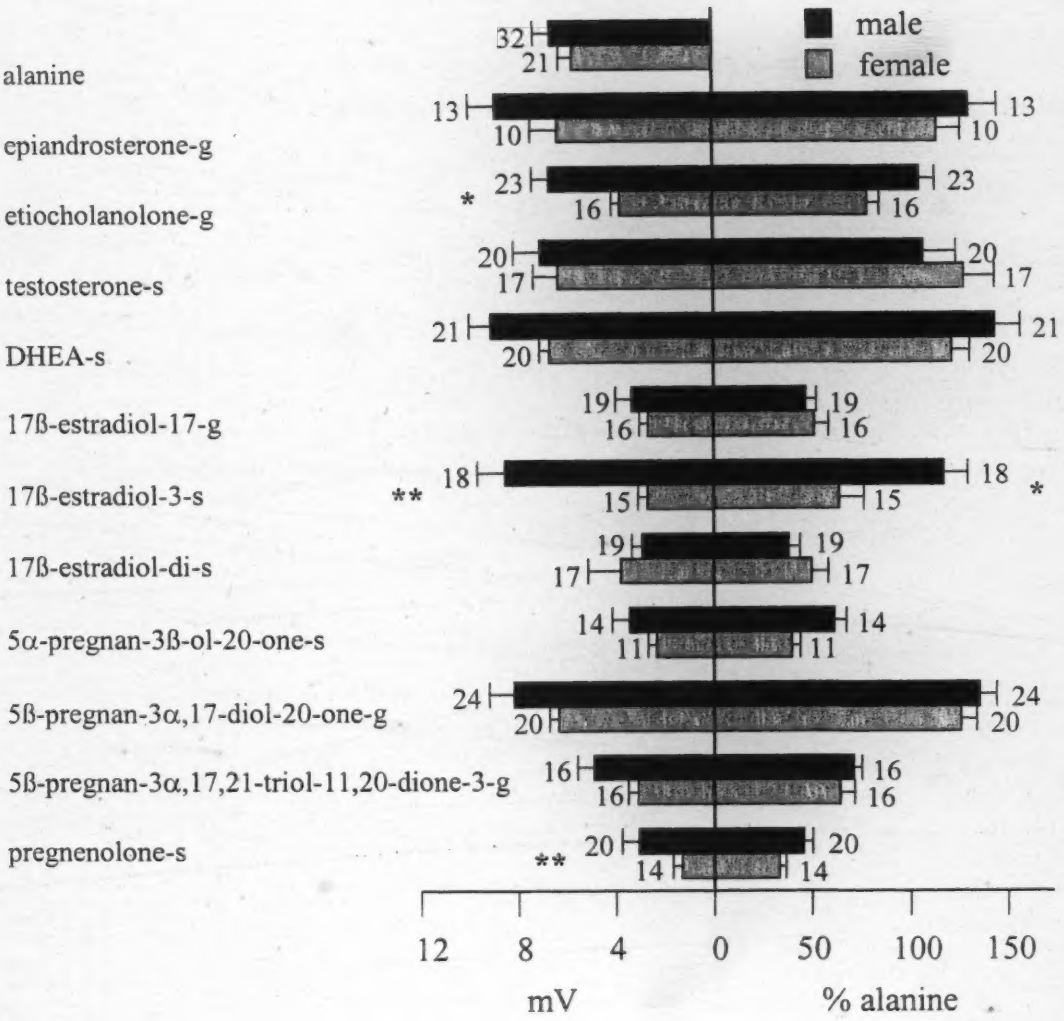

male
female
alanine
epiandrosterone-g
etiocholanolone-g
testosterone-s
DHEA-s
17ß-estradiol-17-g
17ß-estradiol-3-s
17ß-estradiol-di-s
5α-pregnan-3ß-ol-20-one-s
5ß-pregnan-3α,17-diol-20-one-g
5ß-pregnan-3α,17,21-triol-11,20-dione-3-g
pregnenolone-s
32
21
13
10
23
16
20
17
21
20
19
16
18
15
19
17
14
11
24
20
16
16
20
14
*
**
12
8
4
0
50
100
150
mV
% alanine

Figure 2.2a. Concentration response profiles (mean ± S.E.M) for selected C19 and C18 steroids. EOG response expressed as a percentage of that elicited by the amino acid standard (10^{-5} M L-alanine). □ = response of males (N = 5-11); △ = response of females (N = 6).

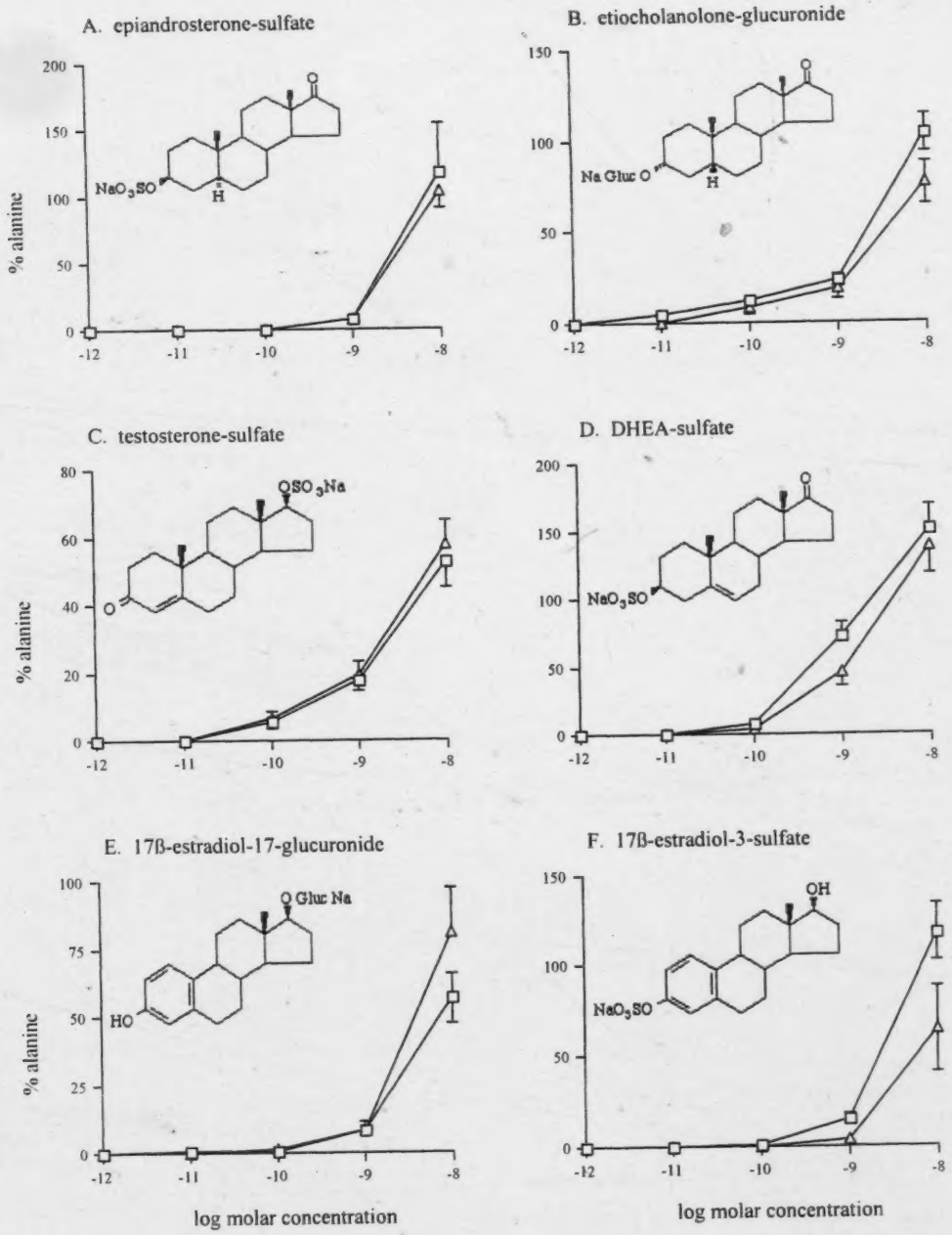
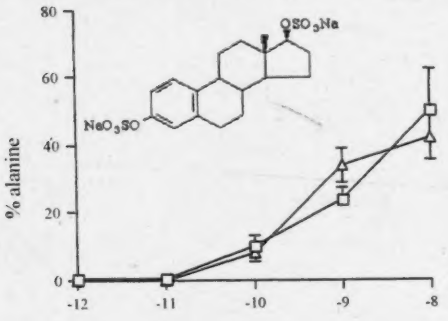
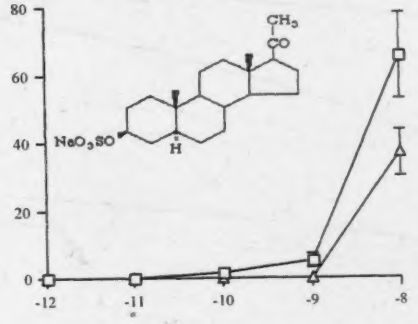

A. epiandrosterone-sulfate
B. etiocholanolone-glucuronide
C. testosterone-sulfate
D. DHEA-sulfate
E. 17ß-estradiol-17-glucuronide
F. 17ß-estradiol-3-sulfate
% alanine
log molar concentration
NaO3SO
Na Gluc O
OSO3Na
O Gluc Na
HO
OH
H
-12
-11
-10
-9
-8

Figure 2.2b. Concentration response profiles (mean ± S.E.M) for selected C18 and C21 steroids. EOG response expressed as a percentage of that elicited by the amino acid standard (10^{-5} M L-alanine). □ = response of males (N = 8-11); Δ = response of females (N = 6).

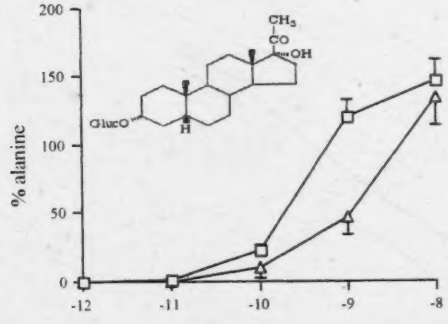
A. 17ß-estradiol-di-sulfate



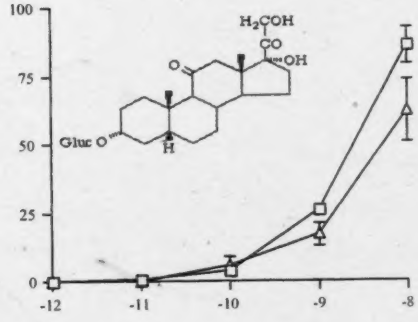
B. 5a-pregnan-3ß-ol-20-one-sulfate



C. 5ß-pregnan-3a,17-diol-20-one-glucuronide



D. 5ß-pregnan-3α,17,21-triol-11,20-dione-3-g



E. pregnenolone-sulfate

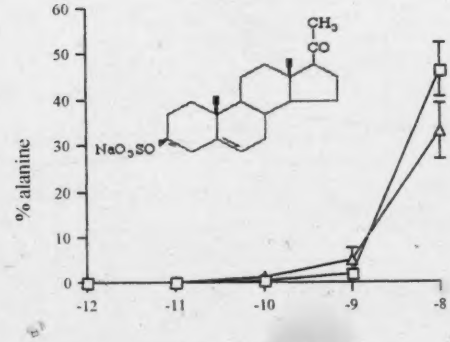
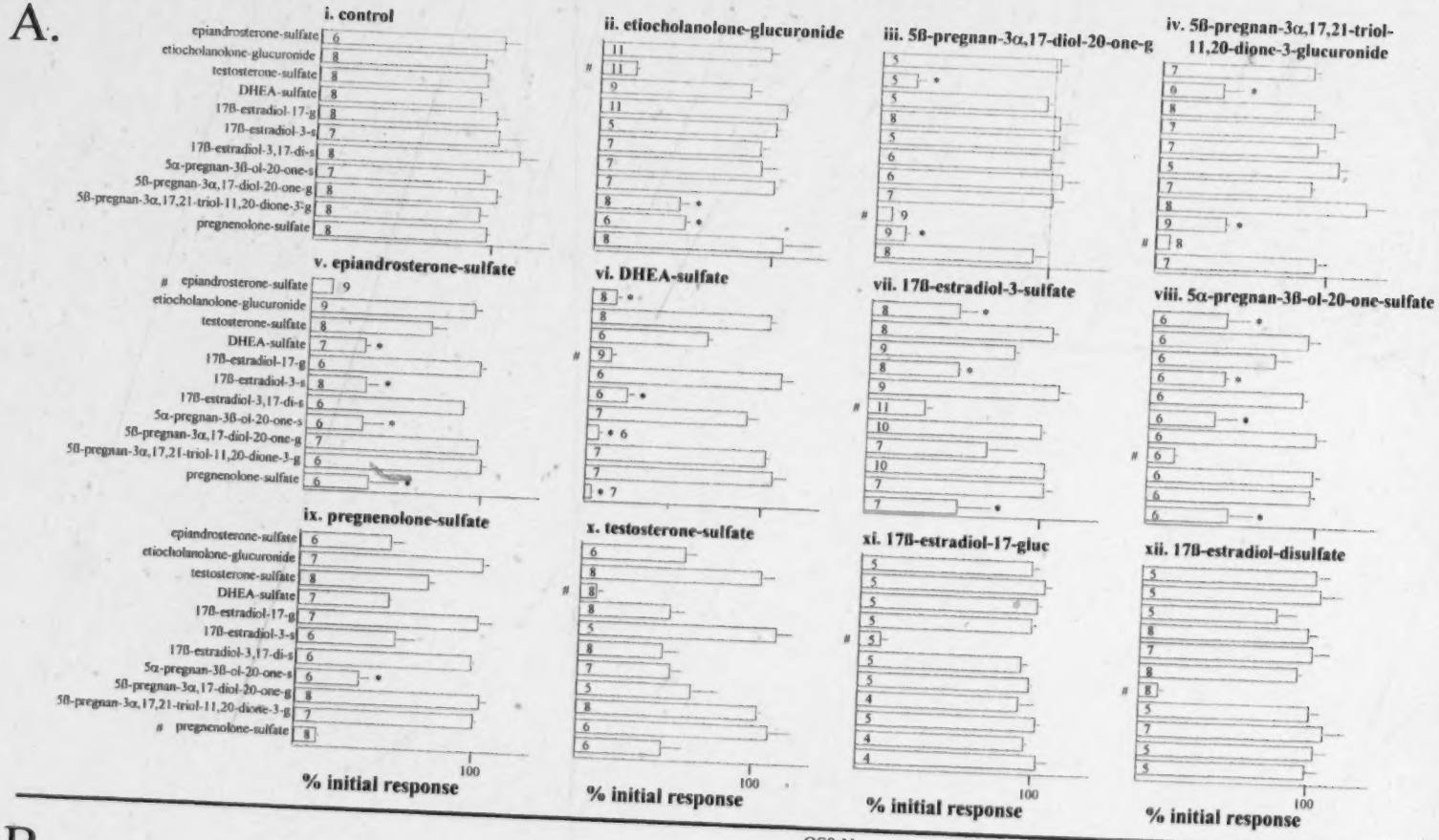


Figure 2.3A. Results of EOG cross-adaptation studies, by adapting stimulus (i-xii). Percent initial response (%IR) was determined by comparing response during adaptation to response given during pre-adaptation. Mean %IR and standard error are shown. Then numbers at the end of the error bars signify sample sizes. Asterisks indicate which steroids act via the same receptor mechanism as the self-adapting control (#) (Dunnett's test, * $P < 0.05$). Control adaptation (sequential exposure) studies (A) show that steroid response was affected by prior exposure.

Figure 2.3B. Schematic representations of each steroid class discriminated by the olfactory epithelium.


A.
i. control
epiandrosterone-sulfate
etiocholanolone-glucuronide
testosterone-sulfate
DHEA-sulfate
17ß-estradiol-17-g
17ß-estradiol-3-s
17ß-estradiol-3,17-di-s
5α-pregnan-3ß-ol-20-one-s
5ß-pregnan-3α,17-diol-20-one-g
5ß-pregnan-3α,17,21-triol-11,20-dione-3-g
pregnenolone-sulfate
ii. etiocholanolone-glucuronide
iii. 5ß-pregnan-3α,17-diol-20-one-g
iv. 5ß-pregnan-3α,17,21-triol-11,20-dione-3-glucuronide
v. epiandrosterone-sulfate
vi. DHEA-sulfate
vii. 17ß-estradiol-3-sulfate
viii. 5α-pregnan-3ß-ol-20-one-sulfate
ix. pregnenolone-sulfate
x. testosterone-sulfate
xi. 17ß-estradiol-17-gluc
xii. 17ß-estradiol-disulfate
% initial response
100

B.

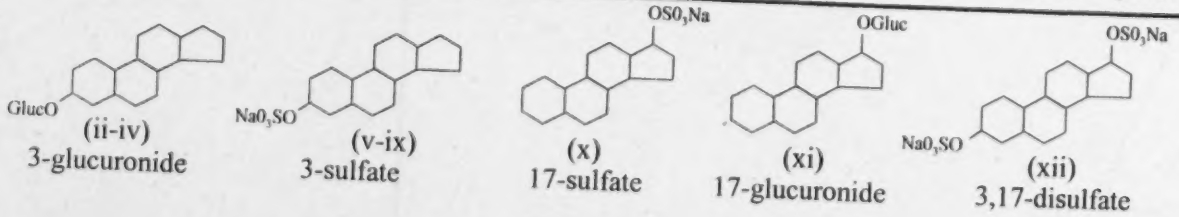

GlucO
(ii-iv)
3-glucuronide
Na0$_3$SO
(v-ix)
3-sulfate
OS0$_3$Na
(x)
17-sulfate
OGluc
(xi)
17-glucuronide
OS0$_3$Na
Na0$_3$SO
(xii)
3,17-disulfate

Figure 2.4. Desensitization of steroid response as seen in binary mixture experiments. Steroids tested once (first exposure), then at twice the initial concentration (2^{nd} exposure), then again at the original concentration (3rd exposure). %IR represents the ratio of 3rd exposure to 1st exposure. A 2.5 minute interval was given between all exposures. Mean %IR ± S.E.M shown, sample sizes adjacent to error bars. % IR was then used to determine the level of additivity expected to occur in binary mixture experiments after desensitization caused by repeated exposure to steroids. * Response was significantly reduced ($P < 0.05$) (Wilcoxon matched-pairs).

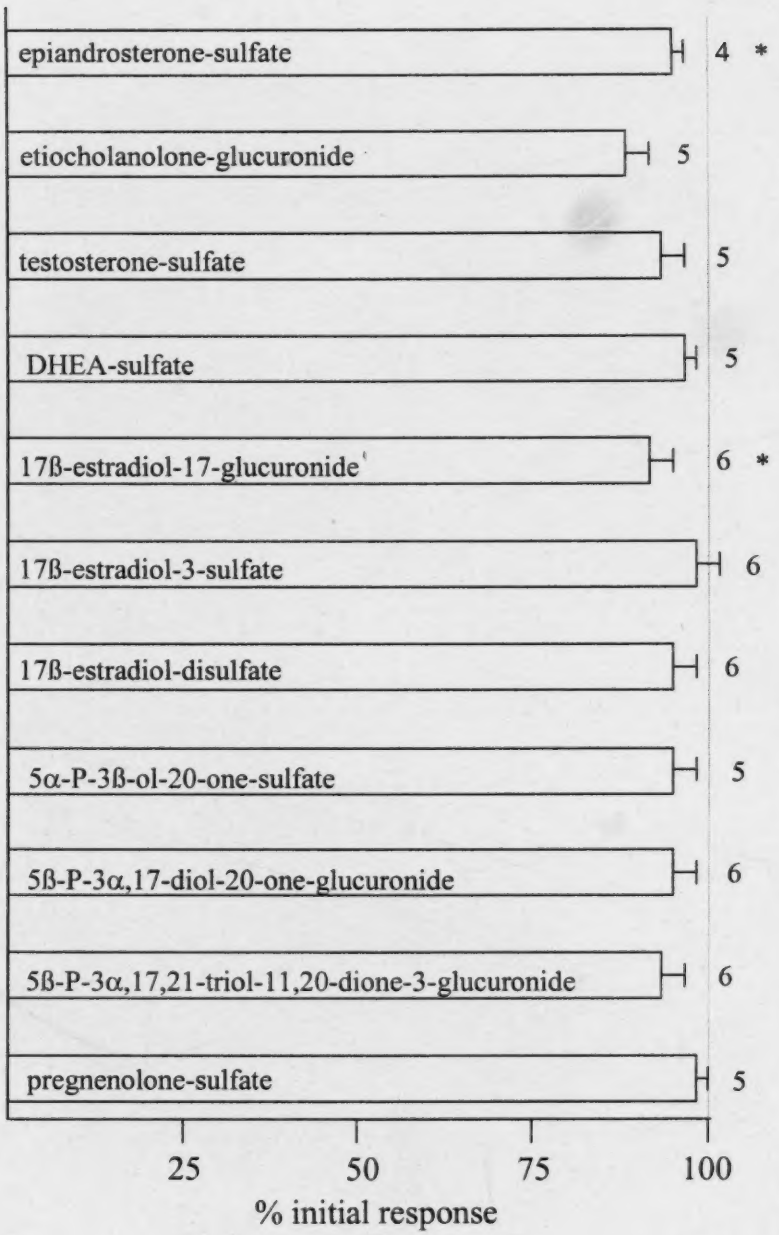

epiandrosterone-sulfate
4 *
etiocholanolone-glucuronide
5
testosterone-sulfate
5
DHEA-sulfate
5
17ß-estradiol-17-glucuronide
6 *
17ß-estradiol-3-sulfate
6
17ß-estradiol-disulfate
6
5α-P-3ß-ol-20-one-sulfate
5
5ß-P-3α,17-diol-20-one-glucuronide
6
5ß-P-3α,17,21-triol-11,20-dione-3-glucuronide
6
pregnenolone-sulfate
5
25
50
75
100
% initial response

Figure 2.5a,b. The independent component index ' (ICI') and mixture discrimination index (MDI) of EOG responses to binary mixtures. The bracket indicates those mixture combinations comprised of components suggested by cross-adaptation to act via the same receptor mechanism (within-group). Bars represent mean ± standard error, $N = 6$ unless otherwise noted. Broken lines indicate predicted ICI' and MDI values for steroids which act via different receptor mechanisms (across-group). Abbreviations used for this figure: epiallopregnanolone-s = 5α-pregnan-3ß-ol-20-one-sulfate; 17α-hydroxypregnanolone-g = 5ß-pregnan-3α,17-diol-20-one-glucuronide; urocortisone-3-g = 5ß-pregnan-3α,17,21-triol-11,20-dione-3-glucuronide.

Figure 2.5c,d. Comparisons of the average (C) independent component index' (ICI'), and (D) mixture discrimination indices of binary mixtures when combined into groups identified by cross-adaptation to represent separate receptor sites. Mean values (± S.E.M) for within-group mixtures (not discriminated) are compared to those of across-group mixtures (discriminated) using a Welch-corrected t-test.

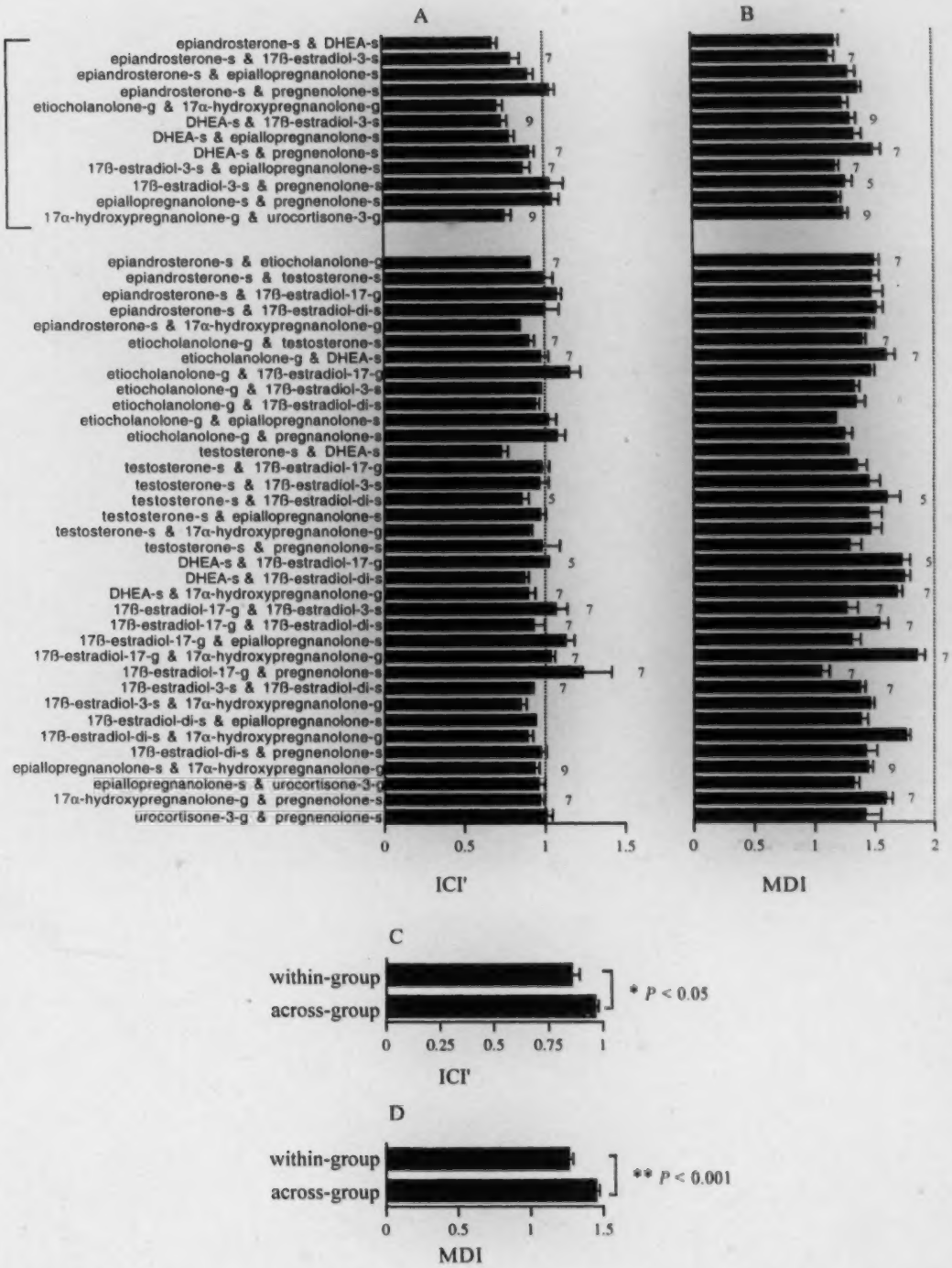

A
B
epiandrosterone-s & DHEA-s
epiandrosterone-s & 17β-estradiol-3-s
epiandrosterone-s & epiallopregnanolone-s
epiandrosterone-s & pregnenolone-s
etiocholanolone-g & 17α-hydroxypregnanolone-g
DHEA-s & 17β-estradiol-3-s
DHEA-s & epiallopregnanolone-s
DHEA-s & pregnenolone-s
17β-estradiol-3-s & epiallopregnanolone-s
17β-estradiol-3-s & pregnenolone-s
epiallopregnanolone-s & pregnenolone-s
17α-hydroxypregnanolone-g & urocortisone-3-g
epiandrosterone-s & etiocholanolone-g
epiandrosterone-s & testosterone-s
epiandrosterone-s & 17β-estradiol-17-g
epiandrosterone-s & 17β-estradiol-di-s
epiandrosterone-s & 17α-hydroxypregnanolone-g
etiocholanolone-g & testosterone-s
etiocholanolone-g & DHEA-s
etiocholanolone-g & 17β-estradiol-17-g
etiocholanolone-g & 17β-estradiol-3-s
etiocholanolone-g & 17β-estradiol-di-s
etiocholanolone-g & epiallopregnanolone-s
etiocholanolone-g & pregnanolone-s
testosterone-s & DHEA-s
testosterone-s & 17β-estradiol-17-g
testosterone-s & 17β-estradiol-3-s
testosterone-s & 17β-estradiol-di-s
testosterone-s & epiallopregnanolone-s
testosterone-s & 17α-hydroxypregnanolone-g
testosterone-s & pregnenolone-s
DHEA-s & 17β-estradiol-17-g
DHEA-s & 17β-estradiol-di-s
DHEA-s & 17α-hydroxypregnanolone-g
17β-estradiol-17-g & 17β-estradiol-3-s
17β-estradiol-17-g & 17β-estradiol-di-s
17β-estradiol-17-g & epiallopregnanolone-s
17β-estradiol-17-g & 17α-hydroxypregnanolone-g
17β-estradiol-17-g & pregnenolone-s
17β-estradiol-3-s & 17β-estradiol-di-s
17β-estradiol-3-s & 17α-hydroxypregnanolone-g
17β-estradiol-di-s & epiallopregnanolone-s
17β-estradiol-di-s & 17α-hydroxypregnanolone-g
17β-estradiol-di-s & pregnenolone-s
epiallopregnanolone-s & 17α-hydroxypregnanolone-g
epiallopregnanolone-s & urocortisone-3-g
17α-hydroxypregnanolone-g & pregnenolone-s
urocortisone-3-g & pregnenolone-s
0
0.5
1
1.5
2
ICI'
MDI
C
within-group
across-group
* P < 0.05
0
0.25
0.5
0.75
1
ICI'
D
within-group
across-group
** P < 0.001
0
0.5
1
1.5
MDI

Table 2.1. All steroids detected by *Haplochromis burtoni*, grouped according to chemical structure. An asterisk (*) indicates which steroids were used in EOG studies.

Chemical Structure	Compound	Common name
C18 estratrienes	1,3,5(10)-estratrien-3α,17β-diol-17-glucuronide*	17β-estradiol-17-glucuronide
	1,3,5(10)-estratrien-3α,17β-diol-3-sulfate*	17β-estradiol-3-sulfate
	1,3,5(10)-estratrien-3α,17β-diol-17-sulfate	17β-estradiol-17-sulfate
	1,3,5(10)-estratrien-3α,17β-diol-3,17-disulfate*	17β-estradiol-3,17-disulfate
	1,3,5(10)-estratrien-3-ol-17-one-sulfate	estrone-3-sulfate
	1,3,5(10)-estratrien-3α,17β-diol-3-sulfate,17-gluc	17β-estradiol-3-sulf,17-gluc
C19 5α-androstan	5α-androstan-3α,17β-diol-17-glucuronide	androsterone-17-glucuronide
	5α-androstan-3α-ol-17-one-3α-glucuronide	androsterone-3-glucuronide
	5α-androstan-3β-ol-17-one-sulfate*	epiandrosterone-sulfate
C19 5β-androstan	5β-androstan-3α-ol-17-one-glucuronide*	etiocholanolone-glucuronide
C19 4-androsten	4-androsten-17β-ol-3-one-sulfate*	testosterone-sulfate
C19 5-androsten	5-androsten-3β-ol-17-one-sulfate*	dehydroepiandrosterone-sulfate
C21 4-pregnen	4-pregnen-20β-ol-3-one-20-sulfate	20β-P-sulfate
C21 5-pregnen	5-pregnen-3β-ol-20-one-sulfate*	pregnenolone-sulfate
C21 5α-pregnan	5α-pregnan-3β-ol-20-one-sulfate*	epiallopregnanolone-sulfate
C21 5β-pregnan	5β-pregnan-3α,17-diol-20-one-glucuronide*	17α-hydroxypregnanolone-gluc
	5β-pregnan-3α,17,21-triol-11,20-dione-3-gluc*	urocortisone-glucuronide

Table 2.2. All steroids used in EOG experiments, grouped according to olfactory receptor classes indicated by cross-adaptation and binary mixture studies.

Class	Compound	Common name
3-sulfate	5α-androstan-3β-ol-17-one-sulfat	epiandrosterone-sulfate
	5-androsten-3β-ol-17-one-sulfate	dehydroepiandrosterone-sulfate
	1,3,5(10)-estratrien-3α,17β-diol-3-sulfate	17β-estradiol-3-sulfate
	5α-pregnan-3β-ol-20-one-sulfate	epiallopregnanalone-sulfate
	5-pregnen-3β-ol-20-one-sulfate	pregnenolone-sulfate
17-sulfate	4-androsten-17β-ol-3-one-sulfate	testosterone-sulfate
3,17-disulfate	1,3,5(10)-estratrien-3α,17β-diol-disulfate	17β-estradiol-3,17-disulfate
3-glucuronide	5β-androstan-3α-ol-17-one-glucuronide	etiocholanolone-glucuronide
	5β-pregnan-3α,17-diol-20-one-glucuronide	17α-hydroxypregnanolone-gluc
	5β-pregnan-3α,17,21-triol-11,20-dione-3-glucuronide	urocortisone-3-gluc
17-glucuronide	1,3,5(10)-estratrien-3α,17β-diol-17-gluc	17β-estradiol-17-glucuronide

P.W. Kluwer Academic, New York.

Sorensen, P.W., Scott, A.P., and Kihslinger, R.L. 2000. How common hormonal metabolites function as relatively specific pheromonal signals in goldfish. pp 125-128. In *Proceedings of the Sixth International Symposium on Reproductive Physiology of Fish.* Edited by Norberg, B., Kjesbu, O.S., Taranger, G.L., Andersson, E., and Stefansson, S.O. John Grieg AS, Bergen.

Sorensen, P.W., Brash, A.R., Goetz, F.W., Kellner, R.G., Bowdin, L., and Vrieze, L.A. 1995a. Origins and functions of F prostaglandins as hormones and pheromones in the goldfish. pp 244-248. In *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish.* Edited by Goetz, F.W. and Thomas, P. FishSymp 95. University of Texas at Austin.

Sorensen, P.W., Scott, A.P., Stacey, N.E., and Bowdin, L. 1995b. Sulfated 17α.20β-dihydroxy-4-pregnen-3-one functions as a potent and specific olfactory stimulant with pheromonal actions in the goldfish. *Gen. Comp. Endocrinol.* **100**: 128-142.

Stacey, N and P.W Sorensen. 2002. Hormonal pheromones in fish. In *Hormones, Brain, and Behavior volume 2.* pp 375-434. Edited by D.W Pfaff, A.P. Arnold, A.M. Etgen, S.E. Fahrbach and R.T. Rubin. Academic Press, San Diego.

Van Den Hurk, R., Schoonen, W.G.E.J., Van Zoelen, G.A., and Lambert, J.G.D. 1987. The biosynthesis of steroid glucuronides in the testis of zebrafish, *Brachydanio rerio,* and their pheromonal function as ovulation inducers. *Gen. Comp. Endocrinol.* **68**: 179-188.

Wong, S.T., Trinh, K., Hacker, B., Chan, G.C.K., Lowe, G., Gaggar, A., Xia, Z, Gold, G.H., and Storm, D.R. 2000. Disruption of the type III adenlylyl cyclase gene leads to peripheral and behavioral anosmia in transgenic mice. *Neuron.* **27**: 487-497.

3. Behavioral Response to Steroid Hormones

3.1 INTRODUCTION

Odors influence a variety of behavioral responses in many vertebrate species. Chemical signals play a particularly important role in intersexual interactions among rodents (Singer et al., 1982, Vandenbergh, 1969) and snakes (Mason et al., 1989). A steroid (5α-androst-16-en-3-one) produced in the testis and released in the saliva of pigs (*Sus scrofa*) is known to facilitate mating by eliciting female receptive behavior (Signoret, 1970; Dorries et al., 1997). Aquatic organisms such as fish provide ample opportunities to examine behavioral responses to chemical cues. The olfactory organ of fish is constantly exposed to chemical information, whereas visual information may often be limited. Aquatic organisms could use released hormones as pheromones because hormones and their metabolites are released to the water bathing the olfactory organ, and provide potentially important information regarding the reproductive status of the releaser.

Kittredge et al. (1971) first provided clear evidence that aquatic organisms may use released hormones as pheromones. Kittredge et al. showed that the female moulting steroid hormone, crustecdysone, has potent effects on male behavior, and suggested that this steroid was likely a mating pheromone in many crustaceans. Since then, the conclusions drawn by Kittredge et al. (1971) have been criticized by many others studying crustacean pheromones (Gleeson et al., 1984). Regardless, the ideas provided by Kittredge et al. have lead to the proposal that many aquatic organisms, including fish, may have evolved hormonal pheromone systems (Doving, 1976). Since then, behavioral assays have aided our understanding of the influence of pheromones on behavior of a number of fish species. To date, there is abundant support for the hormonal pheromone hypothesis in fish (Stacey and Sorensen, 2002; Stacey and Cardwell, 1995) and pheromones have been shown to exert both primer (endocrine) and releaser (behavioral) effects (Sorensen and Stacey, 1999; Stacey and Sorensen, 1986; Stacey et al., 1989).

Soon after the proposal by Doving (1976), behavioral effects of hormonal pheromones were reported in the black goby (*Gobius jozo = Gobius niger*) (Colombo, 1980) and zebrafish (*Danio rerio*) (Van Den Hurk and Lambert, 1983). Black goby females are attracted to both male urine and a 19-carbon steroid, etiocholanolone-glucuronide, which can induce oviposition (Colombo et al., 1980; Colombo et al., 1982). Male zebrafish show greater behavioral response to ovarian extracts of ovulated females than non-ovulated females, and show similar responses to ovarian extracts containing steroid glucuronides, including 17β-estradiol-17-glucuronide and testosterone-glucuronide (Van Den Hurk and Lambert, 1983). However, more recent electro-olfactogram (EOG) studies have yet to provide evidence that zebrafish can physiologically detect these compounds (Stacey et al., 2003). Behavioral assays have since aided our understanding of the influence of pheromones on such important fish taxa as gobiids, salmonids, and cyprinids.

Behavioral response to water-borne hormonal products in goldfish (*Carassius auratus*) has been well documented. 17,20β-P induces final oocyte maturation in female goldfish and, when released into the water, 17,20β-P and a metabolite, 17,20β-P-sulfate, together with androstenedione, function as a preovulatory pheromone evoking specific behavioral responses in males. Males exposed to 17,20β-P exhibit elevated sexual arousal demonstrated by increased swimming and searching behavior (DeFraipoint and Sorenson, 1993). As well, Poling et al. (2001) showed that males exposed to 17,20β-P display low levels of prolonged chasing and nudging behaviors. Stacey and Sorenson (2002) suggest that this mild socio-sexual response may allow males to remain in close proximity to mature females so they have an advantage during ovulation and subsequent spawning. 17,20β-P-sulfate elicits responses similar to those of 17,20β-P, but the chasing and nudging seen in response to 17,20β-P-sulfate exposure are greater and more transient (Poling et al., 2001). Males exposed to androstendione increase aggressive behaviors but do not increase courtship behavior (Poling et al., 2001). Androstenedione is thought to inhibit premature courtship behavior that may be directed towards females that are releasing 17,20β-P in transient pulses early in the periovulatory process (Stacey and Sorenson, 2002; Poling et al., 2001), whereas the elevated aggression shown by males

exposed to androstenedione is believed to be a means of establishing dominance prior to spawning (Poling et al., 2001). The ratio of 17,20β-P to androstenedione increases as oocyte maturation continues, possibly allowing males to identify females closer to ovulation, resulting in more appropriate elevated courtship behavior. Just prior to ovulation, release rates of 17,20β-P-sulfate are maximized (Scott and Sorenson, 1994), resulting in intense courtship. Urinary release of 17,20β-P-sulfate in controlled pulses may be a means by which males can locate specific ovulated females in a group (Poling et al., 2001).

Once mature oocytes move into the oviduct prior to release, synthesis of prostaglandin F2α (PGF2α) stimulates female spawning behavior. PGF2α and a potent metabolite (15-keto-PGF2α) are then released into the water where they function as a postovulatory pheromone to stimulate male courtship behavior. The level of courtship induced by PGF2α is far greater than that of 17,20β-P and groups of males exposed to PGF2α will actively chase and nudge each other (Sorenson et al., 1989). The sexually dimorphic EOG response of goldfish to prostaglandins is of particular note, as mature males show greater sensitivity and response magnitude to PGF2α and 15-keto-PGF2α than do females (Sorensen and Goetz, 1993). This suggests either that prostaglandins are female-specific pheromones used to actively signal to males, or that males are spying on females through passively released prostaglandins (Stacey and Sorensen, 2002). In contrast to the detailed information on hormonal pheromones of goldfish, relatively little is known about hormone-behavior relationships of other fish species, including cichlids (Stacey and Sorensen, 2002).

Immersion in concentrated testosterone (500 μg per L) for several days stimulates aggressive behaviors in a cichlid (*Aequidens pulcher*). Males immersed in testosterone increased agonistic frontal displays and biting behavior directed towards mirrors, models, and other fish (Munro and Pitcher, 1985). These results support earlier studies by Fernald (1976) in which *Haplochromis burtoni* increased approaching and attacking behavior following an intramuscular injection (0.15 mg/g) of testosterone. PGF2α injection in a female cichlid (*Cichlasoma bimaculatum*) rapidly elicits reproductive behaviors including substrate cleaning and simulated

oviposition without egg release (Cole and Stacey, 1984). Studies examining the effects of water-borne PGF2α were not conducted and to date there is no evidence that any species of the Order Perciformes can detect prostaglandin hormones (Robison et al., 1998; Murphy et al., 2001). Although studies of this nature can provide insight into possible roles of hormones in the behavior of cichlids, they do not provide any information about potential hormonal pheromone systems because of the extreme dosages used and lack of evidence that these compounds are detected by the olfactory organ.

This study was conducted to examine the behavioral effects of several individual water-borne steroid odors on *H. burtoni*. The steroids used in odor exposure experiments represent 4 of the 5 receptor mechanisms identified by EOG studies: DHEA-sulfate and pregnenolone-sulfate (3-sulfate); testosterone-sulfate (17-sulfate); 17β-estradiol-3,17-disulfate (3,17-disulfate); 5β-pregnan-3α,17-diol-20-one-glucuronide and etiocholanolone-glucuronide (3-glucuronide) (see Chapter 2). .

Behavioral assays described in this chapter demonstrate that some of these steroids elicit distinct suites of behavioral responses in male *H. burtoni*. These results, coupled with endocrine responses in males exposed to steroid odors (see Chapter 4), indicate the steroid odors detected by *H. burtoni* have biological functions. This research provides the framework for future studies into the hormonal pheromone systems of haplochromines and other cichlids.

3.2 METHODS

Behavioral responses to individual steroids were assayed with odor exposure experiments. One male and two females were allowed 48 hours to acclimate in an 80 L aquarium before detectable steroids were pumped in. Four behaviors were recorded (chasing, courting, sifting through gravel, and overall locomotor activity) over five 10-minute observation periods (pretest, T = 0, T = 10, T = 60, T = 120 minutes). Chasing, courting, and overall activity are behaviors that reflect sexual and aggressive responses, whereas sifting in gravel is a feeding behavior not likely to be mediated through pheromones. Experimental trials were videotaped to minimize

observer effect on fish behavior. The combination of one male and two females made observation of multiple fish performing several behaviors possible, and eliminated male-male aggressive encounters.

3.2.1 Test apparatus

One male and two female cichlids were placed in an 80 L aquarium no less than 48 hours before odor exposure. Aquaria were opaque on both ends and one side, with one side left clear for viewing. Aquaria contained gravel and two terra cotta plant pots (approximately 12 cm diameter) positioned such that the open ends were facing the video camera. Fish were not fed the day of the experiment. Only one odor was tested in each tank per day. Aquaria were kept on constant photoperiod (16L: 8D), with lights coming on at 06:00 hrs. Videotaping occurred between 11:00 and 17:00 hrs. A video camera (Panasonic, PV-L551) on a tripod behind a blind was used to record activity in all areas of the aquaria during the experiments. Odorants were pumped into the test aquarium through silicon tubing using a peristaltic pump (Buchler Instruments, Fort Lee, NJ). The tubing was attached to an airstone in the aquarium to aid in odor dispersion. A dye (food coloring) injection test indicated that odors initially concentrate in a cloud around the airstone and move in pulses around the tank, reaching all corners of the aquarium within 5 minutes.

3.2.2 Test procedure

Approximately two hours before testing, water flow into the test tank was turned off. Videotaping began at the start of the pretest, at which time behavior was observed during the 10 minutes immediately preceding odor addition. After 10 minutes, the peristaltic pump was turned on and 10^{-7} M steroid solution in a 1 L glass bottle was pumped into the test aquarium at 6.5 ml/min for the duration of the test. Behaviors were recorded for ten minute periods beginning at the time of first exposure (T = 0), 10 minutes after first exposure (T = 10), one hour after first exposure (T = 60), and two hours after first exposure (T = 120). This final tank concentration (10^{-9} M) was chosen as all odors used in these experiments have detection thresholds of 10^{-10} M. Fish were expected to be exposed to suprathreshold

odor wisps during odor addition. Fish were not used more than twice in behavioral experiments. When used in more than one behavioral test, a fish was never placed with a fish it had previously been tested with, and was never exposed to the same treatment twice. One treatment (steroid or blank water) was tested in each of the two test aquaria per day and aquaria were then flushed overnight.

Video recordings were viewed to determine the frequency of occurrence of four male behavior patterns (Fernald, 1976; Fernald, 1977): (1) *courtship display*: male quivers sideways near a female with anal fin spread; (2) *chase*: fish rapidly pursues a fleeing opponent; (3) *digging in gravel*: fish bites at gravel and may sift for food (each bite or continuous bout of biting was considered an event); (4) *overall locomotor activity*: estimated activity measure based on total number of line-crossings in a given period. In this measure, the tank was divided into quadrants (by a vertical and horizontal line that bisected the observation side of the aquaria) and a locomotory event consisted of each occurrence in which at least half of a fish's body crossed a quadrant line.

3.2.3 Test steroids

Six steroids (Steraloids, Wilton, NH; Sigma, St. Louis, MO) were used in the behavioral bioassays (etiocholanolone-glucuronide, testosterone-sulfate, DHEA-sulfate, 17ß-estradiol-disulfate, 5ß-pregnan-3α,17-diol-20-one-glucuronide, and pregnenolone-sulfate). These steroids represented 4 of the 5 receptor mechanisms identified by EOG cross-adaptation in Chapter 2 (3-sulfate, 17-sulfate, 3,17-disulfate, and 3-glucuronide). Purchased steroids were dissolved in ethanol, and then 80 µl of 10^{-3} M steroid solution was diluted in 800 ml of water to make the 10^{-7} M test solution. Control treatments for steroid exposure tests contained the same amount of ethanol solvent used for all steroids (80 µl ethanol per 800 ml control test solution). All steroid and control tests were conducted in the same manner.

3.2.4 Data analysis

To determine if steroid odor addition affected cichlid behavior, pretest behavior was compared to responses seen during odor (or control ethanol vehicle) exposure

during all four post-exposure observation periods (T = 0, 10, 60, 120 min) within each treatment using Friedman's nonparametric ANOVA for repeated measures with Dunn multiple comparisons (Instat, GraphPad). Data from the four post-exposure observation periods were also averaged to generate a measure of average behavioral events recorded after odor exposure began. Average behaviors recorded in these four observation periods were compared (Wilcoxon tests; Instat, GraphPad) to pretest responses to determine overall behavioral response without time constraints.

3.3 RESULTS

3.3.1 Behavioral responses

During the pre-test period, none of the four behavioral measures differed among groups (Kruskal-Wallis ANOVA, $P > 0.05$). Although behaviors of males exposed to a control ethanol vehicle did not change throughout the testing procedure, some behaviors did change as a result of exposure to steroid odors.

When exposed to 17ß-estradiol-3,17-disulfate, male courtship behavior increased ($P = 0.003$), most notably at T = 60 min (Figure 3.1). Further, the median courtship seen in all four post-exposure intervals was significantly greater than in the pretest interval ($P = 0.03$). There was a slight trend of increased chasing (Figure 3.2), and a general trend ($P = 0.055$) for males exposed to 17ß-estradiol-3,17-disulfate to increase gravel digging behavior (Figure 3.4). Courtship, chasing, and overall locomotor activity appear to decline by the final observation period (T = 120), whereas responses to control odor addition remained stable.

Males exposed to 5ß-pregnan-3α,17-diol-20-one-glucuronide increased overall locomotor activity ($P = 0.049$) (Figure 3.3), although no individual post-exposure period was different than the pre-test period. Median total chasing behavior of males exposed to 5ß-pregnan-3α,17-diol-20-one-glucuronide approached significance ($P = 0.08$; Figure 3.2). A trend for males to increase courtship in response to 5ß-pregnan-3α,17-diol-20-one-glucuronide was observed; however, these differences were not significant (Figure 3.1). Male gravel digging behavior was unaffected by 5ß-pregnan-3α,17-diol-20-one-glucuronide exposure ($P > 0.05$). Although not as

pronounced as in the 17ß-estradiol-disulfate studies, a trend for reduced behavioral response at the final observation period (T = 120 min) was seen for courtship displays, chasing, and locomotor activity.

Exposure to etiocholanolone-glucuronide increased chasing behavior ($P = 0.014$), most notably at T = 10 ($P < 0.05$) and T = 120 ($P < 0.05$) (Figure 3.2). The median number of chasing events over all post-exposure observation periods was significantly different than pretest responses ($P = 0.016$). The courtship ANOVA for etiocholanolone-glucuronide-exposed males approached significance ($P = 0.07$; Figure 3.1).

Males exposed to testosterone-sulfate increased overall locomotor activity ($P = 0.024$), most notably at T = 10 ($P < 0.05$) (Figure 3.3) and also increased median chasing behavior ($P = 0.016$) (Figure 3.2). The reduction in behavioral responsiveness at the final observation period as seen in 17β-estradiol-3,17-disulfate and 5ß-pregnan-3α,17-diol-20-one-glucuronide exposure studies is not as pronounced here. Behaviors observed during DHEA-sulfate exposure remained stable throughout the testing procedure, and no significant increases from pretest conditions were observed. Similarly, pregnenolone-sulfate exposure did not stimulate any behavioral changes in males (data not shown).

3.4 DISCUSSION

Behavioral assays suggest that some detected steroids have behavioral functions in *H. burtoni*. Further, different steroids induced different suites of behaviors, which is expected among steroids that act via separate receptor mechanisms. 17β-estradiol-3,17-disulfate exposed males increased courtship behavior. Males exposed to testosterone-sulfate increased both overall locomotor activity and chasing behavior. Males exposed to 5β-pregnan-3α,17-diol-20-one-glucuronide increased overall locomotor activity and show a trend for increased chasing behavior. Etiocholanolone-glucuronide, a steroid that acts via the same receptor mechanism as 5β-pregnan-3α,17-diol-20-one-glucuronide, stimulated an increase in chasing behavior. Males exposed to an ethanol control vehicle exhibited no response and

behaviors in this group did not change significantly throughout the testing procedure. DHEA-sulfate and pregnenolone-sulfate exposed males did not exhibit behavioral response. The lack of behavioral response to water-borne DHEA-sulfate is similar to results seen in another perciform fish, the round goby (Murphy and Stacey, 2002). which also exhibits EOG response to this steroid.

There was no apparent relationship between steroid exposure and sifting through gravel behavior. Although it has been reported that male *H. burtoni* dig spawning pits in the gravel (Fernald, 1977), this behavior was rarely observed during general observations and never seen in behavioral bioassays. All bites directed towards the gravel in these steroid-exposure experiments are believed to be feeding attempts. although there was a trend for median gravel sifting in all four post-observation intervals to increase in 17β-estradiol-3,17-disulfate exposed males ($P = 0.055$).

17β-estradiol-3,17-disulfate, 5β-pregnan-3α,17-diol-20-one-glucuronide, and eticholanolone-glucuronide are excellent candidates for pheromones in *H. burtoni*. First, since 17β-estradiol is produced by the growing ovary and is known to regulate vitellogenesis (Specker and Sullivan, 1994), one may expect plasma (and in turn urine) levels of estrogens to increase as females near ovulation, providing nearby males with a clear signal of mature vitellogenic females that are close to ovulation. Plasma levels of 17β-estradiol have been shown to be higher in females than in males of another cichlid, *Oreochromis mossambicus* (Rocha and Reis-Henriques, 1996). In addition, a number of estrogens are detected by the olfactory epithelium of *H. burtoni*, some of which were not included for examination in this study. The three estrogens I focused on in these experiments are all detected through separate receptor mechanisms, implicating them as potentially important players in the chemical communication system of *H. burtoni*. Further, male EOG response threshold to 17β-estradiol-3,17-disulfate was among the lowest of all steroids examined.

The C21 steroid 5β-pregnan-3α,17-diol-20-one-glucuronide is reported to attract female African catfish (*Clarias gariepinus*) (Resink et al., 1989). Analysis of holding water from mature male and female catfish suggests that this steroid is released in sufficient amounts to be detectable (Van Weerd et al., 1991). EOG results in this study showed that 5β-pregnan-3α,17-diol-20-one-glucuronide is detected with

remarkable sensitivity (10^{-10} M) by *H. burtoni* and is the second most potent olfactory stimulant (EOG response magnitude) in this species for both males and females. Although previous studies have shown this steroid to affect only female behavior (Resink et al., 1989), it is possible that the regime used in the present study prevented such results. The combination of one male and two females may not have provided the social context necessary to elicit behavioral responses in females. Additional cues not present in these experiments may be required by *H. burtoni* to induce behavioral responses. Social context and status have been shown to modulate behavior, sex steroid concentration, and growth rates in cichlids (Hofmann et al., 1999; Oliveira et al., 1996). It is also possible that males show such sensitivity and response magnitude to 5β-pregnan-3α,17-diol-20-one-glucuronide as a spying tactic, responding to signals released by nearby males that may have attracted an ovulating female.

Etiocholanolone-glucuronide has been implicated as a possible pheromone in another perciform species, the black goby (Colombo et al., 1980), where it is thought to attract females to nest sites for oviposition. Free etiocholanolone, which acts through the same receptor mechanism as etiocholanolone-glucuronide in the round goby, also has pheromonal function and increases ventilation rate in both males and females (Murphy and Stacey, 2002). Murphy and Stacey (2002) suggested that etiocholanolone may have similar functions in the round goby as its glucuronated conjugate has in the black goby, facilitating female mate location. Further, the response of round goby males to water-borne etiocholanolone suggests potential pheromonal interaction among males.

Although individually tested steroids elicited behavioral responses in this study, it is possible that mixes or blends of steroids may play an important role in *H. burtoni*. Steroids released by fish are expected to occur as complex mixtures, with component ratios changing throughout the reproductive cycle. This phenomenon is best understood in the goldfish preovulatory pheromone, where the ratio of components drastically influences the effect of the signal (Sorensen et al., 1998). Although I did not attempt to quantify behavioral responses to steroid mixtures, experiments reported

in chapter 4 suggest pheromonal mixtures may be important in inducing endocrine response in male *H. burtoni*.

2.6 LITERATURE CITED

Brunet, L.J., Gold, G.H., and Ngai, J. 1996. General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. *Neuron.* **17**: 681-693.

Caprio, J. and Byrd, R.P., Jr. 1984. Electrophysiological evidence for acidic, basic, and neutral amino acid olfactory receptor sites in catfish. *J. Gen. Physiol.* **84**: 403-422.

Caprio, J., Durek, J., and Robinson, J.J., II. 1989. Electro-olfactogram and multiunit olfactory responses to binary and trinary mixtures of amino acids in the channel catfish, *Ictalurus punctatus. J. Gen. Physiol.* **93**: 245-262.

Cardwell, J.R., Stacey, N.E., Tan, E.S.P., McAdam, D.S.O., and Lang, S.L.C. 1995. Androgen increases olfactory receptor response to a vertebrate sex pheromone. *J. Comp. Physiol A.* **176**: 55-61.

Colombo, L., Marconato, A., Belvedere, P.C., and Frisco, C. 1980. Endocrinology of teleost reproduction: A testicular steroid pheromone in the black goby, *Gobius jozo* L. *Boll. Zool.* **47**: 355-364.

Daniel, P.C. and Derby, C.D. 1991. Mixture suppression in behavior: the antennular flick response in the spiny lobster toward binary odorant mixtures. *Physiol. Behav.* **49**: 591-601.

Dulka, J.G., Stacey, N.E., Sorensen, P.W., and Van Der Kraak, G.J. 1987. A steroid sex pheromone synchronizes male-female spawning readiness in goldfish. *Nature.* **325**: 251-253.

Fernald, R.D. 1977. Quantitative behavioral observations of *Haplochromis burtoni* under semi-natural conditions. *Anim. Behav.* **25**: 643-653.

Hosoya, Y. and Yashida, H. 1937. Über de bioelektrische Erscheinung an der Riechschleimhaut. *Jpn. J. Med. Sci. Biol. III. Biophys.* **5**: 22-23.

Jachner, A. 1995. Chemical communication in fish. *Wiadomsci Ekologiczne.* **41**: 153-175.

Johansen, P.H. 1985. Female pheromone and the behavior of male guppies (*Poecilia reticulata*) in a temperature gradient. *Can. J. Zool.* **63**: 1211-1213.

Kang, J. and Caprio, J. 1991. Electro-olfactogram and multiunit olfactory receptor responses to complex mixtures of amino acids in the channel catfish *Ictalurus punctatus*. *J. Gen. Physiol.* **98**: 699-721.

Li, W. and Sorensen, P.W. 1997. Highly independent olfactory receptor sites for naturally occurring bile acids in the sea lamprey, *Petromyzon marinus*. *J. Comp.* Physiol. A. **180**: 429-438.

Lower, N.A., Scott, A.P., and Moore, A. Release of sex steroids into the water by roach (*Rutilus rutilus*). *J. Fish. Biol.* (In press).

Murphy, C.A., Stacey, N., and Corkum, L.D. 2001. Putative steroidal pheromones in the round goby, *Neogobius melanostomus*: olfactory and behavioral responses. *J. Chem. Ecol.* **27**: 443-470.

Nakanishi, S. 1995. Second order neurones and receptor mechanisms in visual- and olfactory-information processing. *TINS.* **18**: 359-364.

Olson, K.S., and Derby, C.D. 1995. Inhibition of taurine and 5′AMP olfactory receptor sites of the spiny lobster *Punulirus argus* by odorant compounds and mixtures. *J. Comp. Physiol. A.* **176**: 527-540.

Ottoson, D. 1956. Analysis of the electrical activity of the olfactory epithelium. *Acta. Physiologica. Scandinavica.* **35** (supp 122): 1-83.

Poling, K.R., Fraser, E.J., and Sorensen, P.W. 2001. The three steroidal components of the goldfish preovulatory pheromone signal evoke different behaviors in males. *Comp. Biochem. Physiol. B.* **129**: 645-651.

Robison, R.R., Fernald, R.D., and Stacey, N.E. 1998. The olfactory system of a cichlid fish responds to steroidal compounds. *J. Fish. Biol.* **53**: 226-229.

Scott, J.W., and Scott-Johnson, P.E. 2002. The electoolfactogram: A review of its history and uses. *Microsc. Res. Tech.* **58**: 152-160.

Sorensen, P.W., and Caprio, J. 1997. Chemoreception. pp 375-405. In *The Physiology of Fishes, 2nd edtion*. Edited by D.H. Evans. CRC Press, Boca Raton, FL.

Sorensen, P.W., and Stacey, N.E. 1999. Evolution and specialization of fish hormonal pheromones. pp 15-47. In *Advances in Chemical Signals in Vertebrates*. Edited by Johnson, R.E. and Müller-Schwarze, D., and Sorensen,

Figure 3.2. Male chasing behavior in the 10 min prior to (pre) and during four 10-min intervals following exposure to a steroid (N = 7-8) or a control ethanol vehicle (N = 10). Box plots show median (bar in the middle), 25th and 75th percentiles (lower and upper end of the box), and absolute range (whiskers). Median chases during all four post-exposure observation intervals (total) are presented to the right. An asterisk (*) represents a significant difference ($P < 0.05$) from pretest.

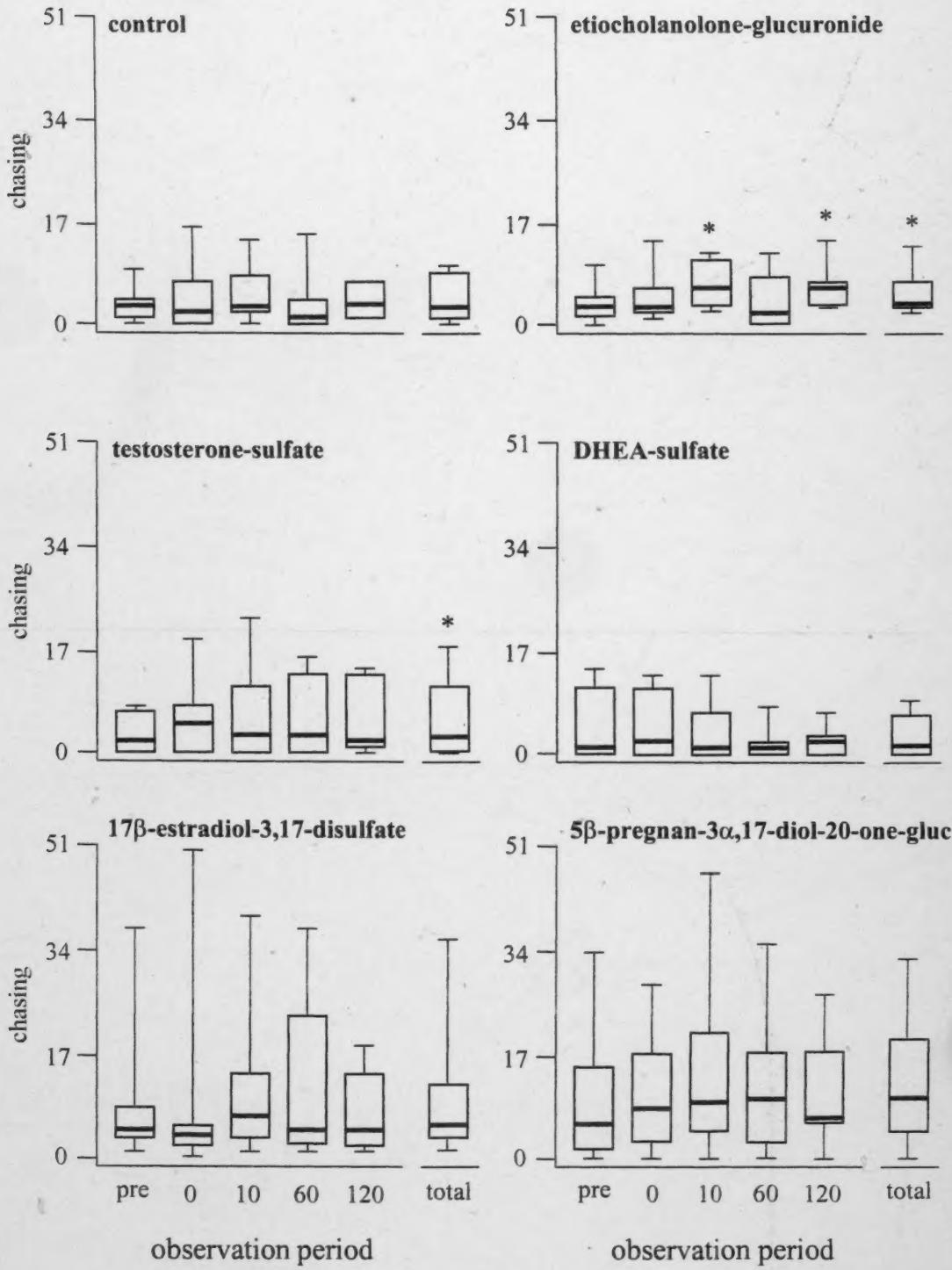

control
etiocholanolone-glucuronide
testosterone-sulfate
DHEA-sulfate
17β-estradiol-3,17-disulfate
5β-pregnan-3α,17-diol-20-one-gluc
chasing
51
34
17
0
*
pre
0
10
60
120
total
observation period

Figure 3.3. Male overall locomotor activity in the 10 min prior to (pre) and during four 10-min intervals following exposure to a steroid (N = 7-8) or a control ethanol vehicle (N = 10). Box plots show median (bar in the middle), 25th and 75th percentiles (lower and upper end of the box), and absolute range (whiskers). Median activity during all four post-exposure observation intervals (total) are presented to the right. An asterisk (*) represents a significant difference ($P < 0.05$) from pretest.

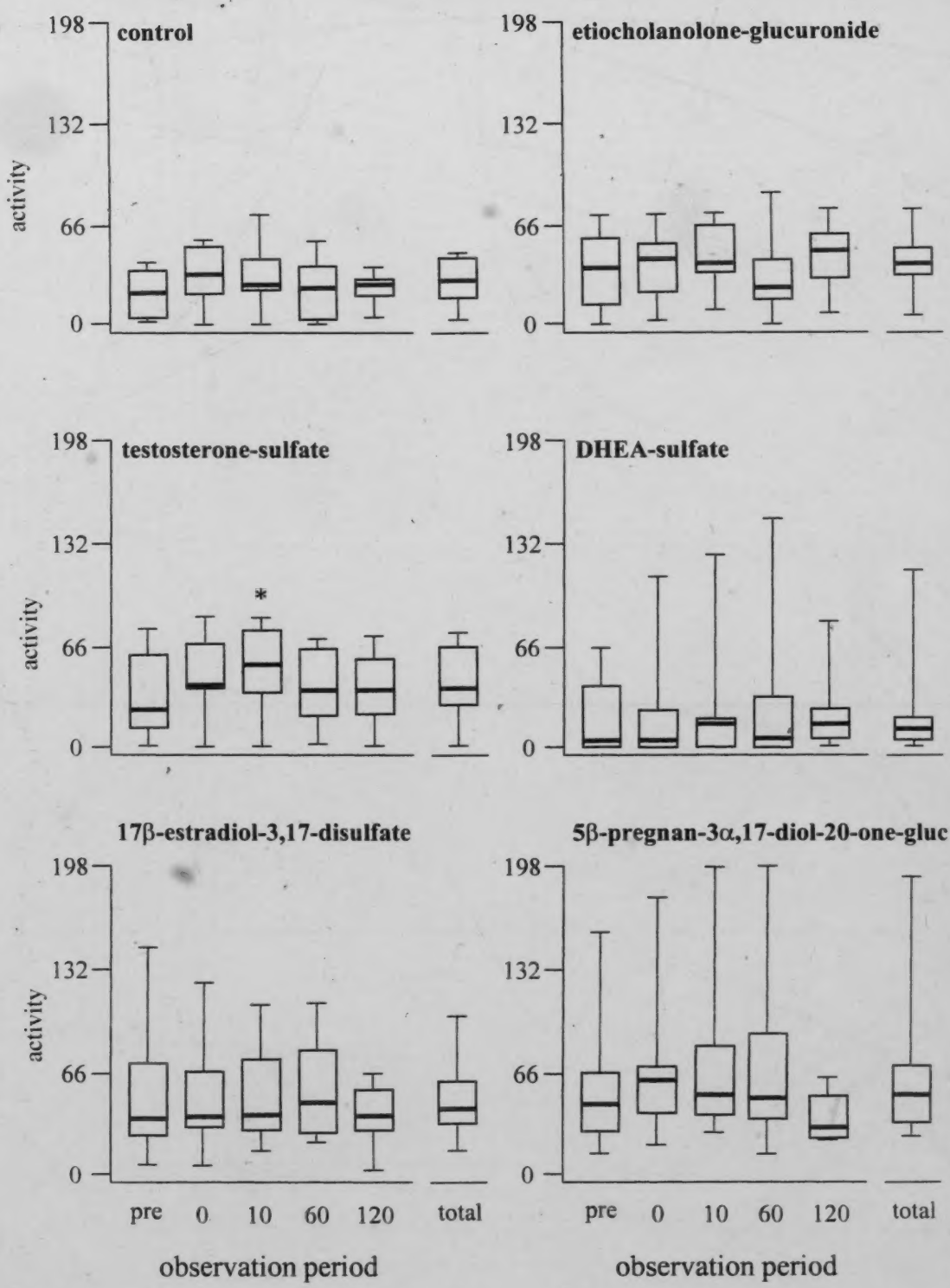
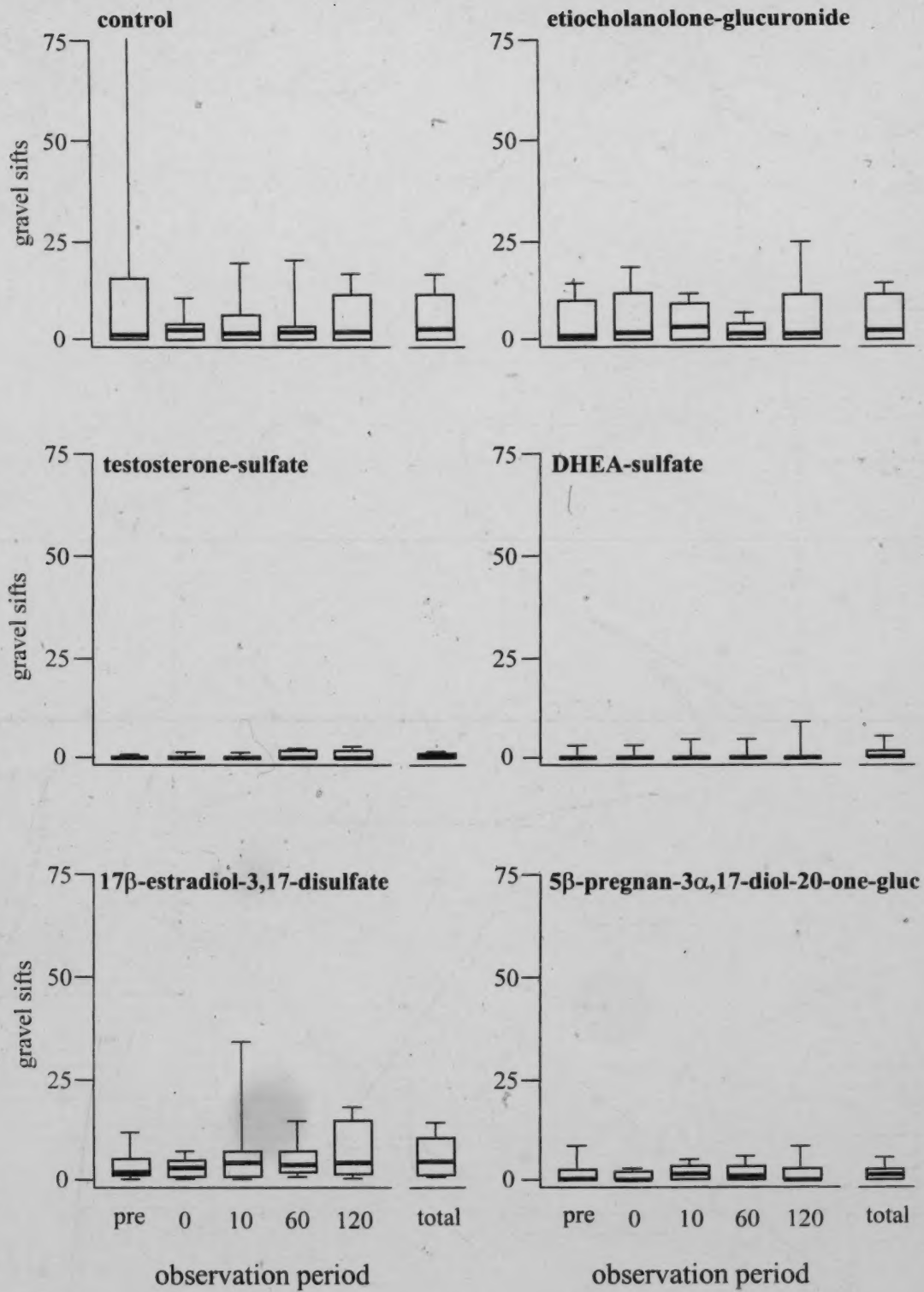

control
etiocholanolone-glucuronide
testosterone-sulfate
DHEA-sulfate
17β-estradiol-3,17-disulfate
5β-pregnan-3α,17-diol-20-one-gluc
activity
198
132
66
0
*
pre
0
10
60
120
total
observation period

Figure 3.4. Male gravel sifting behavior in the 10 min prior to (pre) and during four 10-min intervals following exposure to a steroid (N = 7-8) or a control ethanol vehicle (N = 10). Box plots show median (bar in the middle), 25th and 75th percentiles (lower and upper end of the box), and absolute range (whiskers). Median gravel digs during all four post-exposure observation intervals (total) are presented to the right.


control
etiocholanolone-glucuronide
testosterone-sulfate
DHEA-sulfate
17β-estradiol-3,17-disulfate
5β-pregnan-3α,17-diol-20-one-gluc
gravel sifts
75
50
25
0
pre
0
10
60
120
total
observation period

3.5 LITERATURE CITED

Cardwell, J.R. and Stacey, N.E., Tan, E.S.P., McAdam, D.S.O., and Lang, S.L.C. 1995. Androgen increases olfactory receptor response to a vertebrate sex pheromone. *J. Comp. Physiol A.* **176**: 55-61.

Cole, K.S. and Stacey, N.E. 1984. Prostaglandin induction of spawning behavior in *Cichlasoma bimaculatum* (Pisces Cichlidae). *Horm. Behav.* **18**: 235-248.

Colombo, L., Marconato, A., Belvedere, P.C., and Frisco, C. 1980. Endocrinology of teleost reproduction: A testicular steroid pheromone in the black goby, *Gobius* jozo L. Boll. Zool. **47**: 355-364.

Colombo, L., Belvedere, P.C., Marconato, A., and Bentivegna, F. 1982. Endocrinology of teleost reproduction. pp 84-94. In *Proceedings of the Second International Symposium on the Reproductive Physiology of Fish.* Edited by C.J.J. Richter and H.J.T. Goos editors. Pudoc, Netherlands.

DeFraipoint, M. and Sorensen, P.W. 1993. Exposure to the pheromone 17α,20β-dihydroxy-4-pregnen-3-one enhances the behavioral spawning success, sperm production, and sperm motility of male goldfish. *Anim. Behav.* **46**: 245-256.

Dorries, K. M., Adkins-Regan, E., and Halpern, B.P. 1997. Sensitivity and behavioral responses to the pheromone androstenone are not mediated by the vomeronasal organ in domestic pigs. *Brain Behav. Evol.* **49**, 53-62.

Doving, K. 1976. Evolutionary trends in olfaction. pp 149-159. In *The Structure-Activity Relationships in Olfaction.* Edited by G. Benz. IRL Press, London.

Fernald, R.D. 1976. The effect of testosterone on the behavior and coloration of adult male cichlid fish (*Haplochromis burtoni*). *Horm. Res.* **7**: 172-178.

Fernald, R.D. 1977. Quantitative behavioral observations of *Haplochromis burtoni* under semi-natural conditions. *Anim. Behav.* **25**: 643-653.

Gleeson, R.A., Adams, M.A., and Smith, A.B. 1984. Characterization of a sex pheromone in the blue crab, *Callinectes sapidus*: Crustecdysone studies. *J. Chem. Ecol.* **10**: 913-921.

Hofmann, H.A., Benson, M.E., and Fernald, R.D. 1999. Social status regulates growth rate: Consequences for life history strategies. *Proc. Natl. Acad. Sci.*

96: 14171-14176.

Kittredge, J.S., Terry, M., and Takahashi, F.T. 1971. Sex pheromone activity of the moulting hormone, crustecdysone, on male crabs *Pachygrapsus crassipes, Cancer antennarius,* and *C. anthonyi. Fish. Bull.* **69**: 337-343.

Mason, R.T., Fales, H.M., Jones, T.H., Pannell, L.K., Chinn, J.W., and Crews, D. 1996.Sex pheromones in snakes. *Science.* **245**: 290-293.

Munro, A.D and Pitcher, T.J. 1985. Steroid hormones and antagonistic behavior in a Cichlid teleost, *Aequidens pulcher. Horm. Behav.* **19**: 353-371.

Murphy, C.A., Stacey, N., and Corkum, L.D. 2001. Putative steroidal pheromones in the round goby, *Neogobius melanostomus*: olfactory and behavioral responses. *J. Chem. Ecol.* **27**: 443-470.

Oliveira, R.F., Almada, V.C., and Canario, A.V. 1996. Social modulation of sex steroid concentrations in the urine of male cichlid fish *Oreochromis mossambicus. Horm. Behav.* **30**: 2-12.

Poling, K.R., Fraser, E.J., and Sorensen, P.W. 2001. The three steroidal components of the goldfish preovulatory pheromone signal evoke different behaviors in males. *Comp. Biochem. Physiol. B.* **129**: 645-651.

Resink, J.W., Voorthius, P.K., Van Den Hurk, R., Peters, R.C., and Van Oordt, P.G.W.J. 1989. Steroid glucuronides of the seminal vesicle as olfactory stimuli in African catfish *Clarias gariepinus. Aquaculture.* **83**: 153-166.

Robison, R.R., Fernald, R.D., and Stacey, N.E. 1998. The olfactory system of a cichlid fish responds to steroidal compounds. *J. Fish. Biol.* **53**: 226-229.

Rocha, M.J. and Reis-Henriques, M.A. 1996. Plasma and urine levels of C_{18}, C_{19}, and C_{21} steroids in an asynchronous fish, the tilapia *Oreochromis mossambicus* (Teleostei, Cichlidae). *Comp. Biochem. Physiol. C.* **115**: 257-264.

Scott, A.P. and Sorensen, P.W. 1994. Time course of release of pheromonally active steroids and their conjugates by ovulatory goldfish. *Gen. Comp. Endocrinol.* **96**: 309-323.

Signoret, J.P. 1970. Reproductive behavior of pigs. *J. Reprod. Fert. Suppl.* **11**: 105-107.

Singer, A.G., Macrides, F., and Agosta, W.C. 1982. Studies of hamster reproductive

pheromones. pp 365-375. In *Chemical Signals, Vertebrates and Aquatic Invertebrates*. Edited by D. Muller-Schwarze and R.M. Silverstein. Plenum Press, New York, London.

Sorensen, P.W., and Stacey, N.E. 1999. Evolution and specialization of fish hormonal pheromones. pp 15-47. In *Advances in Chemical Signals in Vertebrates*. Edited by R.E. Johnson and D. Müller-Schwarze, D. and P.W. Sorensen. Kluwer Academic, New York.

Sorensen, P.W., Chamberlain, K.J., and Stacey, N.E. 1989. Differing behavioral and endocrinological effects of two female sex pheromones on male goldfish. *Horm. Behav.* **23**: 317-332.

Sorensen, P.W. and Goetz, F.W. 1993. Pheromonal and reproductive function of F-prostaglandins and their metabolites in teleost fish. *J. Lipid Mediators.* **6**: 385-393.

Sorensen, P.W., Christensen, T.A., and Stacey, N.E. 1998. Discrimination of pheromonal cues in fish: emerging parallels with insects. *Curr. Opin. Neurobiol.* **8**: 458-467

Specker, J.L. and Sulivan, C.V. 1994. Vitellogenesis in fishes: status and perepectives. In Perspectives in Endocrinology. pp 304-315. Edited by K.G Davey, R.E. Peter, and S.S. Tobe. National Research Council, Ottawa.

Stacey, N and P.W Sorensen. 2002. Hormonal pheromones in fish. pp 375-434. In *Hormones, Brain, and Behavior, volume 2.* Edited by D.W. Pfaff, A.P. Arnold, A.M. Etgen, S.E. Fahrbach and R.T. Rubin. Academic Press, San Diego.

Stacey, N.E., and Cardwell, J.R. 1995. Hormones as sex pheromones in fish: Widespread distribution among freshwater species. pp 244-248. In *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish.* Edited by F.W. Goetz and P. Thomas. FishSymp 95. University of Texas, Austin.

Stacey, N.E., Sorensen, P.W., Van Der Kraak, G.J., and Dulka, J.G. 1989. Direct evidence that 17α,20β-dihydroxy-4-pregnen-3-one functions as a goldfish primer pheromone: Preovulatory release is closely associated with male endocrine responses. *Gen. Comp. Endocrinol.* **75**: 62-70.

Stacey, N.E. and Sorensen, P.W. 1986. 17α,20β-dihydroxy-4-pregnen-3-one: A steroidal primer pheromone which increases milt volume in the goldfish. *Can. J. Zool.* **64**: 2412-2417.

Stacey, N., Chojnacki, A., Narayanan, A., Cole, T., and Murphy. C. 2003. Hormonally derived sex pheromones in fish: exogenous cues and signals from gonad to brain. *Can. J. Physiol. Pharmacol.* **81**: 329-341.

Vandenbergh, J.G. 1969. Male odor accelerates female sexual maturation in mice. *Endocrinology*. **84**: 658-660.

Van Den Hurk, R., and Lambert, J.G.D. 1983. Ovarian steroid glucuronides function as sex pheromones for male zebrafish, *Brachydanio rerio*. *Can J. Zool.* **61**: 2381-2387.

Van Weerd, J.H., Sukkel, M., Lambert, J.G.D., and Richter, C.J.J. 1991. GCMS-identified steroids and steroid glucuronides in ovarian growth-stimulating holding water from adult African catfish, *Clarias gariepinus*. *Comp. Biochem. Physiol.* **98**: 303-311.

4. Endocrine Response to Steroid Hormones

4.1 INTRODUCTION

Although some fish sex pheromones have dramatic behavioral effects, evoking a range of responses including courtship, aggression, predator aversion, migration, and individual recognition (see Stacey and Sorenson, 2002; Liley, 1982 for reviews), others elicit physiological effects in fish that are less conspicuous but just as significant. In the well studied goldfish, *Carassius auratus*, the oocyte maturation-inducing steroid 17,20β-P is released into the water where it functions as a preovulatory pheromone. Male goldfish exposed to water-borne 17,20β-P increase blood gonadotropin II (GtH-II) levels within 15 minutes and increase milt volume within 6 hours (Dulka et al., 1987; Stacey et al., 1989). Similarly, the postovulatory pheromone PGF2α (and metabolites) also has physiological effects, causing a rapid increase in male GtH-II concentration and milt volume (Kyle et al., 1985). Pheromonal 17,20β-P also significantly increases sperm motility in goldfish (DeFraipoint and Sorenson, 1993; Zheng et al., 1997). This elevation in sperm motility likely increases male reproductive fitness, as 17,20β-P-exposed males fertilize more eggs than control males in both pair-spawning and group-spawning situations (Zheng et al., 1997). Pheromonal PGF2α stimulates GtH-II release and increases milt volume in brown trout (*Salmo trutta*) (Olsen et al., 2000). There is evidence that prostaglandins may elicit endocrine responses in Atlantic salmon (*Salmo salar*) (Moore and Waring, 1996). Males exposed to urine from ovulated female Atlantic salmon show increases in plasma GtH-II, 17,20β-P, and testosterone (Waring et al., 1996).

This study was designed to evaluate possible endocrine effects of steroid hormones known to stimulate the olfactory epithelium of *H. burtoni*. I first examined the effects of exposure to a mixture of water-borne steroids on serum testosterone concentration of individual *H. burtoni* males. Components of this mixture were then tested individually to determine the effects of single water-borne steroids on male serum testosterone concentration. The results of this study demonstrate a biological

effect of detected steroid hormones, confirming the usefulness of EOG studies in identifying putative pheromonal compounds.

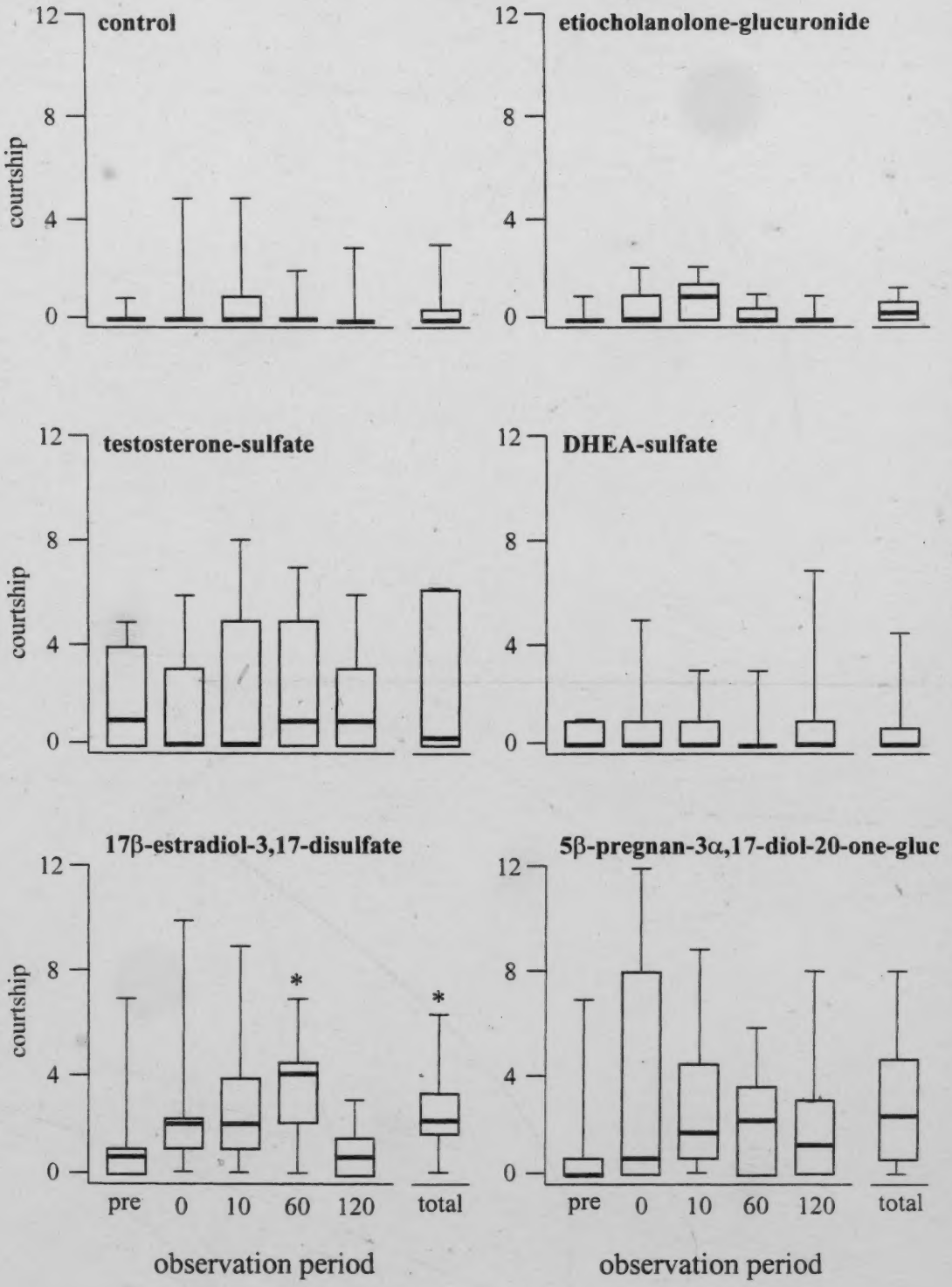
4.2 METHODS

The same group of male *H. burtoni* (all the adult males in the breeding colony) were used in three experiments involving steroid exposure and blood sampling. Individual males were not tracked through the three experiments, and were randomly assigned to treatment groups.

Experiment 1.

Adult male *H. burtoni* were placed individually in 80 L aquaria that contained a bed of gravel and a terra cotta plant pot as shelter. Tanks were kept at 25^0 C under a constant photoperiod (14:10). Fish were allowed 48 hours to acclimate to these conditions before being exposed to a mixture of steroids representing all 5 proposed receptor mechanisms ($N = 10$) or an ethanol control ($N = 10$). Eighty µL each of 10^{-3} M testosterone-sulfate, dehydroepiandrosterone-sulfate (DHEA-sulfate), 17β-estradiol-3,17-disulfate, and 5ß-pregnan-3α,17-diol-20-one-glucuronide and 800 µL of 17β-estradiol-17-glucuronide were added to all experimental aquaria. The final tank concentration of each odor was 10^{-9} M, except for 17β-estradiol-17-glucuronide which was presented at 10^{-8} M because of its higher olfactory detection threshold. For the control test, the amount of ethanol solvent in the steroid mixture (680 µL) was added to each aquarium. Odors were added as a bolus injection by syringe near the airstone to aid in dispersal. After 45-60 min exposure, fish were netted and anesthetized in 0.05 % TMS (Syndel). Blood was taken by syringe from the caudal vasculature. Fish were then weighed, and recovered from anesthesia within 2 minutes. Blood was stored at 4^0 C for 2 hours before serum was extracted. Serum was then stored at -20^0 C and assayed in duplicates using a Testosterone EIA kit (ICN Pharmacuticals) within 24 hours of collection. Data were analyzed using a two-tailed Mann-Whitney test (InStat, GraphPad).

Figure 3.1. Male courtship behavior in the 10 min prior to (pre) and during four 10-min intervals following exposure to a steroid (N = 7-8) or a control ethanol vehicle (N = 10). Box plots show median (bar in the middle), 25th and 75th percentiles (lower and upper end of the box), and absolute range (whiskers). Median courtship displays during all four post-exposure observation intervals (total) are presented to the right. An asterisk (*) represents a significant difference ($P < 0.05$) from pretest.


control
etiocholanolone-glucuronide
testosterone-sulfate
DHEA-sulfate
17β-estradiol-3,17-disulfate
5β-pregnan-3α,17-diol-20-one-gluc
courtship
12
8
4
0
*
pre
0
10
60
120
total
observation period

(Figure 4.2). One component of the steroid mixture (17β-estradiol-17-glucuronide) was not tested individually, and it is possible that this steroid was responsible for the increased serum testosterone shown by males exposed to the 5 steroid mixture. Pheromonal mixtures or blends may play an integral role in this endocrine response, and single steroid odors may not be sufficient to elicit an endocrine response. It is also possible that these individually tested steroids have no endocrine effect on males, or that their effects are behavioral rather than endocrine. It is important to note two key differences between experiment 1 and experiments 2 and 3 that may account for these different results. Blood samples were taken 45-65 minutes after odor exposure in experiment 1, and 120 minutes after odor exposure in experiments 2 and 3. It is possible that the serum testosterone increase is rapid and transient, and any endocrine effect may not be seen by 120 minutes post-exposure. As well, fish in experiments 2 and 3 were isolated in their test aquaria for much longer time periods than in experiment 1. It is unclear what effect isolation may have on *H. burtoni*, but it may be possible that males will not respond to water-borne steroids without recent (within 48 hours) visual contact with other fish.

Steroids can have rapid behavioral effects (Stacey and Sorensen, 2002), perhaps even fast enough that males can have a steroid hormone-induced behavioral change during a normal courtship or aggressive interaction. Social interactions can affect circulating androgen levels in a number of teleost fish including *H. burtoni* (Oliveira et al., 2002). Territorial male *H. burtoni* have higher serum testosterone and 11-ketotestosterone levels than non-territorial males (Francis and Fernald, 1993). Another male cichlid, *Oreochromis mossambicus*, increases circulating 11-ketotestosterone in response to courtship (Borges et al., 1998). In rainbow trout, *Oncorhynchus mykiss*, behavioral effects (chasing) and endocrine changes are closely linked. When subordinate male *O. mykiss* become dominant, they significantly increase aggressive behavior and plasma levels of testosterone and 17,20β-P in comparison to remaining subordinates within 24 hours (Cardwell et al., 1996). Female rainbow trout release compound(s) in the urine that stimulate male 17,20β-P and GtH-II levels (Scott et al., 1994; Scott and Liley, 1994). It is possible that newly dominant males have greater access to females and pheromones released than

subordinates, and this increased odor exposure is quickly followed by a change in circulating hormones. This is supported by studies showing that in the presence of ovulating females, anosmic male *O. mykiss* have lower levels of sex steroids and milt production than control males (Rouger and Liley, 1993).

More studies are required to provide a clearer understanding of the effects of both single steroid odors (17β-estradiol-17-glucuronide in particular) and odor mixtures on serum steroid hormone concentrations. In particular, it would be interesting to determine if detected steroids induce endocrine effects in female *H. burtoni*.

Figure 4.1. Mean serum testosterone (± S.E.M) of males exposed to a 10-9 M mixture of steroids representing the most potent ligand in each receptor class. The asterisk indicates a significant difference from control ethanol vehicle ($P = 0.015$).

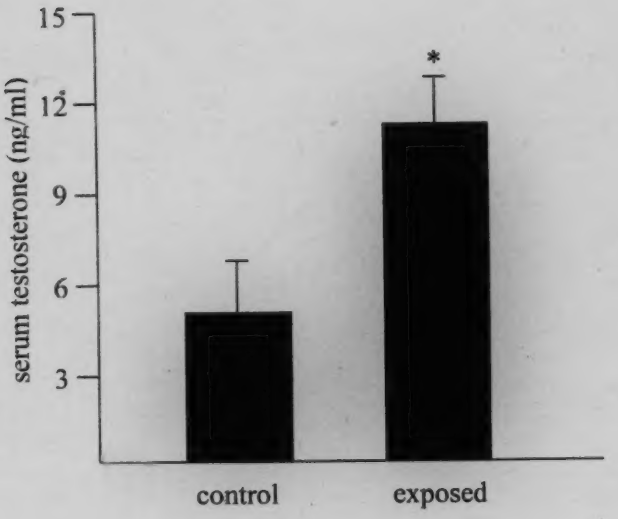
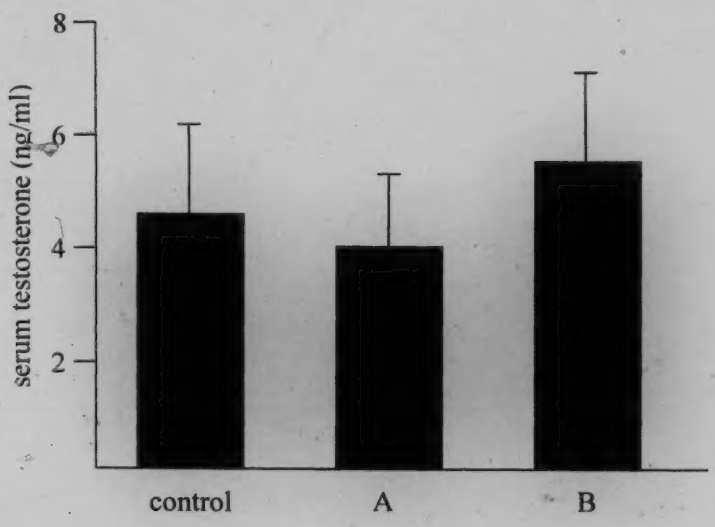
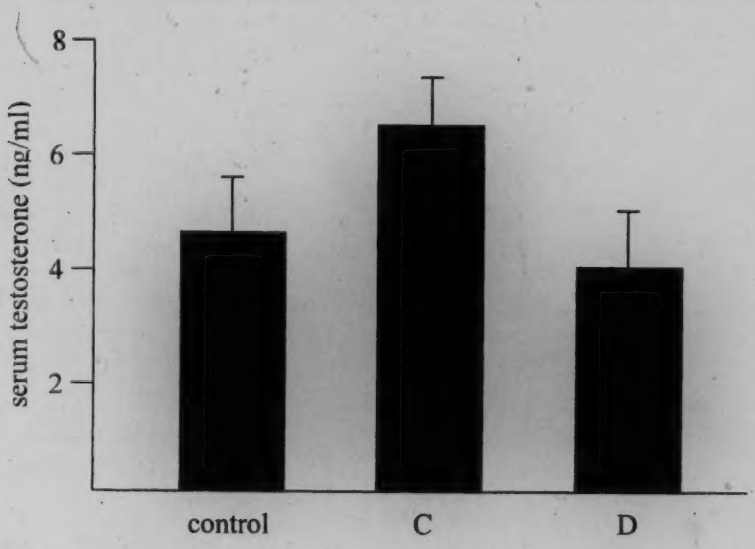

15
12
9
6
3
serum testosterone (ng/ml)
*
control
exposed

Figure 4.2. Mean serum testosterone (± S.E.M) of males exposed to 10^{-9} M A) 5β- pregnan-3α-17-diol-20-one-glucurunide, B) DHEA-sulfate, C) testosterone-sulfate, D) 17β-estradiol-3,17-disulfate, or an ethanol control vehicle.


serum testosterone (ng/ml)
8
6
4
2
control
A
B


serum testosterone (ng/ml)
8
6
4
2
control
C
D

4.5 LITERATURE CITED

Borg, B. 1994. Androgens in teleost fishes. *Comp. Biochem. Physiol. C.* **109**: 219-245.

Borges, R.A., R.F. Oliveira, V.C. Almada, and A.V.M. Canario. 1998. Short-term social modulation of 11-ketotestosterone urinary levels in males of the cichlid fish *Oreochromis mossambicus* during male-female interaction. *Acta. Ethol.* **1**: 43-48.

Cardwell, J.R., P.W. Sorensen, G.J. Van Der Kraak, and N.R. Liley. 1996. Effect of dominance status on sex hormone levels in laboratory and wild-spawning male trout. *Gen. Comp. Endocrinol.* **101**: 333-341.

DeFraipoint, M. and Sorensen, P.W.. 1993. Exposure to the pheromone 17α,20β-dihydroxy-4-pregnen-3-one enhances the behavioral spawning success, sperm production, and sperm motility of male goldfish. *Anim. Behav.* **46**: 245-256.

Dulka, J.G., Stacey, N.E., Sorensen, P.W., and Van Der Kraak, G.J. 1987. A steroid sex pheromone synchronizes male-female spawning readiness in goldfish. *Nature.* **325**: 251-253.

Francis, R.C. and R.D. Fernald. 1993. Neuroendocrine effects of dominance status in an African cichlid fish, *Haplochromis burtoni*. *Z. Tierpsychol.* **60**: 66-82.

Kyle, A.L., N.E. Stacey, R.E. Peter, and R. Billard. 1985. Elevations in gonadotropin concentrations and milt volumes as a result of spawning in the goldfish. *Gen. Comp. Endocrinol.* **57**:10-22.

Liley, N.R. 1982. Chemical communication in freshwater fish. *Can. J. Fish. Aquatic. Sci.* **39**: 22-35.

Moore, A. and C.P. Waring. 1996. Electrophysiological and endocrinological evidence that F-series prostaglandins function as priming pheromones in mature male Atlantic salmon (*Salmo salar*) parr. *J. Exp. Biol.* **199**: 2307-2316.

Oliveira, R.F., Hirschenhauser, K., Carneiro, L.A., and Canario, A.V.M. 2002. Social modulation of androgen levels in male fish. *Comp. Biochem. Physiol. B.* **132**: 203-215.

Olsen, K.H., R. Bjerselius, E. Petersson, E. Jarva, I. Mayer, and M. Hedenskog. 2000. Lack of species-specific primer effects of odours from female Atlantic

salmon, *Salmo salar*, and brown trout, *Salmo trutta*. *Oikos*. **88**: 213-220.

Rouger, Y. and N.R. Liley. 1993. Effect of social environment on plasma hormones and availability of milt in spawning male rainbow trout *Oncorhynchus mykiss* (Walbaum). *Can. J. Zool.* **71**: 280-285.

Scott, A.P. and N.R. Liley. 1994. Dynamics of excretion of 17α,20β-dihydroxy-4-pregnen-3-one-20-sulphate, and of the glucuronids of testosterone and 17β-oestradiol, by urine of reproductively mature male and female rainbow trout (*Oncorhynchus mykiss*). *J. Fish. Biol.* **44**: 117-129.

Scott, A.P., N.R. Liley, and E.L.M. Vermeirssen. 1994. Pheromonal effects of female urine and of synthetic 17α,20β-dihydroxy-4-pregnen-3-one-20-sulphate on plasma sex steroid levels, gonadotrophin-II levels, and milt volumes, in male rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish. Biol.* **44**: 131-147.

Sorensen, P.W., Scott, A.P., and Kihslinger, R.L. 2000. How common hormonal metabolites function as relatively specific pheromonal signals in goldfish. pp 125-128. In *Proceedings of the Sixth International Symposium on the Reproductive Physiology of Fish*. Edited by B. Norberg, O.S. Kjesbu, G.L. Taranger, E. Andersson, and S.O. Stefansson. John Greig AS, Bergen.

Stacey, N and P.W. Sorensen. 2002. Hormonal pheromones in fish. pp 375-434. In *Hormones, Brain, and Behavior volume 2.* Edited by D.W. Pfaff, A.P. Arnold, A.M. Etgen, S.E. Fahrbach and R.T. Rubin. Academic Press, San Diego.

Stacey, N.E., P.W. Sorensen, G.J Van Der Kraak, and J.G. Dulka. 1989. Direct evidence that 17α,20β-dihydoxy-4-pregnen-3-one functions as a goldfish primer pheromone: preovulatory release is closely associated with male endocrine responses. *Gen. Comp. Enocrinol.* **75**: 62-70.

Waring, C.P., and A. Moore. 1996. Milt and endocrine responses of mature male Atlantic salmon (*Salmo salar* L.) parr to water-borne testosterone, 17,20β-dihydroxy-4-pregnen-3-one-20-sulfate, and the urines from adult female and male salmon. *Gen. Comp. Endocrinol.* **103**: 142-149.

Zheng, W. and N.E. Stacey. 1996. Two mechanisms for increasing milt volume in male goldfish, *Carassius auratus*. *J. Exp. Zool.* **276**: 287-295.

Zheng, W., C. Strobeck, and N.E. Stacey. 1997. The steroid pheromone 17α,20β-

dihydoxy-4-pregnen-3-one increases fertility and paternity in goldfish. *J. Exp. Biol.* **200**: 2833-2840.

5. Discussion

This research, which employed EOG studies, behavioral bioassays, and enzyme immunoassays, is perhaps the most elaborate and detailed attempt to characterize the chemical communication system of a perciform fish. The results provide novel evidence that an African cichlid fish, *Haplochromis burtoni*, detects and responds to water-borne conjugates of steroid hormones. These results demonstrate the importance of chemical communication in *H. burtoni,* which can be added to the growing list of fish species thought to employ hormonal pheromone systems. The olfactory epithelium of *H. burtoni* is able to detect a variety of conjugated steroids that act through 5 independent receptor mechanisms, but does not detect unconjugated steroids or prostaglandins. Behavioral bioassays showed that several steroids elicit different responses in males. Further, male *H. burtoni* increase serum testosterone concentrations when exposed to a mixture of water-borne steroids representing all five known olfactory receptor mechanisms. This endocrine response suggests that the ability to detect steroids via the olfactory epithelium is adaptive, and reinforces the importance of EOG studies in attempts to examine hormonal pheromone systems among fish. The results of this study have provided insight into the hormonal pheromone systems of cichlid fish, and represent a first step in developing potential culture and management applications for this vulnerable group of fish.

5.1 Olfactory response to steroid hormones

EOG studies show that the olfactory epithelium of *H. burtoni* is sensitive to a number of steroid hormones (Figure 2.1). These results are in marked contrast to those seen in EOG studies of another cichlid, *Oreochromis* mossambicus, which does not detect any of a number of steroids tested (Frade et al., 2002). This is the only evidence to date that there may be differences in detection of social odors among African cichlids. EOG cross-adaptation experiments indicate that steroids detected by *H. burtoni* act through 5 independent olfactory receptor mechanisms, which can be classified by conjugate type and position: 3-sulfate, 17-sulfate, 3,17-disulfate, 3-

glucuronide, 17-glucuroide (Figure 2.3b). Binary mixture experiments support these cross-adaptation groupings (Figure 2.5). It is of particular interest to note that the three estrogens used in this study all act through separate receptor mechanisms. This result implicates estrogens as potentially important chemical cues. It also suggests that the release ratio of these steroids may convey vital information to the receiver, as seen in goldfish where two very similar compounds, 17,20β-P and 17,20β-P-sulfate also act independently and exert distinct effects (Scott and Sorenson, 1994; Sorensen et al., 2000; Poling et al., 2001).

Two potential problems were observed in binary mixture tests that should be considered in future studies. First, slight desensitization of EOG response was seen during binary mixture testing. The mechanism responsible for this response desensitization remains unclear but I believe that the test procedure itself, which includes presentation of steroids in increasing concentration, is at least partly responsible. Olfactory receptor neurons may require more time between exposures to regain their full binding capabilities. This response desensitization may be the result of temporary second-messenger pathway degradation. The increased ionic permeability of olfactory receptor neurons is responsible for depolarizing the receptor potential, providing the EOG response. Ca^{2+} ion influx gated by cAMP is believed to depolarize the olfactory receptor neuron in freshwater (Leveteau et al., 1989; Laberge and Hara, 2001). There is evidence that calcium amplifies the EOG response by increasing chloride conductance (Frings et al., 2000). It is possible that the supply of integral compounds involved in signal transduction cascades (e.g cAMP or Ca^{2+}) is exhausted by the end of the test procedure. This is unlikely because there was no such response desensitization seen during cross-adaptation experiments, which include presentation of many more odor pulses than binary mixtures. This phenomenon has not been observed in previous binary mixture studies, but Murphy et al. (2001) did show a degradation of EOG response during repeated exposures to steroids presented 30 minutes apart. To avoid this response desensitization, test odors should be presented at the greatest intervals possible during binary mix tests. Additionally, as was done in this study, desensitization factors should be calculated

for each steroid of interest and used to determine the appropriate level of additivity expected to occur.

The second potential problem with binary mixture studies is addressed in Appendix 2. Although the EOG response curves generated by different odors can vary, with peak magnitudes occurring at slightly different times, binary mixture test analysis examines the additive effect of two odors through calculations that assume the response of a mixture will be equal to the sum of the responses generated by each of the independent components. Since the two components involved in the test may induce peak amplitude at different times, it is unreasonable to assume that two odors that act via separate receptor mechanisms will show complete additivity when combined as a mixture. This important issue should be considered when attempting to establish receptor mechanisms responsible for olfactory responses. Despite these potential problems, binary mixtures are a very good means of examining the discriminatory capabilities of the olfactory epithelium. Cross-adaptation coupled with binary mixture studies can provide an extensive characterization of the olfactory organ and both techniques should be employed when possible.

5.2 Behavioral response to steroid hormones

Male *H. burtoni* exhibit behavioral response to four steroids, 17β-estradiol-3,17-disulfate (increased courtship), testosterone-sulfate (increased chasing and overall locomotor activity), 5β-pregnan-3α,17-diol-20-one-glucuronide (increased overall locomotor activity), and etiocholanolone-glucuronide (increased chasing). Males did not respond behaviorally to DHEA-sulfate, pregnenolone-sulfate, or the control ethanol vehicle exposure. The lack of behavioral response to DHEA-sulfate has been observed in another perciform fish, the round goby (Murphy et al., 2001). Despite being a potent olfactory stimulant in the round goby and *H. burtoni*, the pheromonal function of this steroid remains undetermined. There was no correlation between steroid exposure and gravel sifting, a behavior likely unrelated to reproduction.

While behavioral response of control groups remained constant throughout odor exposure tests, courtship, chasing, and overall locomotor activity responses of 17β-

Experiment 2.

Test conditions, procedure, and analysis in experiment 2 were identical to those of experiment 1 unless otherwise noted. Fish were allowed 144 hours to acclimate to tank conditions before being exposed to a single steroid odor (DHEA-sulfate ($N = 11$) or 5ß-pregnan-3α,17-diol-20-one-glucuronide ($N = 12$)) or a control ethanol vehicle ($N = 10$). Eighty µL each of 10^{-3} M DHEA-sulfate, 5ß-pregnan-3α,17-diol-20-one-glucuronide, or control ethanol were added to experimental aquaria. The final tank concentration of each odor was 10^{-9} M. Blood was taken from the caudal vasculature 2 hours after odor exposure, fish were weighed and recovered within 2 minutes.

Experiment 3.

Test conditions, procedure, and analysis in experiment 3 were identical to those of experiment 1 unless otherwise noted. Fish were allowed 50 days to acclimate to tank conditions before being exposed to a single steroid odor (testosterone-sulfate ($N = 10$) or 17β-estradiol-3,17-disulfate ($N = 9$)) or a control ethanol vehicle ($N = 11$). Eighty µL each of 10^{-3} M testosterone-sulfate, 17β-estradiol-3,17-disulfate, or control ethanol were added to experimental aquaria. The final tank concentration of each odor was 10^{-9} M. Blood was taken from the caudal vasculature 2 hours after odor exposure, fish were weighed and recovered within 2 minutes.

4.3 RESULTS

Experiment 1.

Male *H. burtoni* exposed to a mixture of steroids representing all 5 proposed receptor mechanisms had significantly greater serum testosterone concentrations than control males ($P = 0.015$; Figure 4.1). The body weight (mean ± S.E.M.) of males exposed to the steroid mixture (12.2 ± 1.2) was not significantly different ($P > 0.05$) than the body weight of control males (15.5 ± 2.5) (t-test).

Experiment 2.

Serum testosterone concentrations of male *H. butoni* exposed to either DHEA-sulfate or 5ß-pregnan-3α,17-diol-20-one-glucuronide did not differ from males exposed to a control ethanol vehicle ($P > 0.05$; Figure 4.2). The body weights (mean ± S.E.M) of males exposed to DHEA-sulfate (13.4 ± 0.9) and 5ß-pregnan-3α,17-diol-20-one-glucuronide (14.4 ± 1.0) were not significantly different ($P > 0.05$) than the body weight of control males (14.3 ± 0.9) (Kruskal-Wallis ANOVA).

Experiment 3.

Serum testosterone concentrations of male *H. burtoni* exposed to either testosterone-sulfate or 17β-estradiol-3,17-disulfate did not differ from males exposed to a control ethanol vehicle ($P > 0.05$; Figure 4.2). The body weights (mean ± S.E.M) of males exposed to testosterone-sulfate (15.1 ± 0.8) and 17β-estradiol-3,17-disulfate (16.9 ± 2.5) were not significantly different ($P > 0.05$) than the body weight of control males (16.7 ± 1.8) (Kruskal-Wallis ANOVA).

4.4 DISCUSSION

These results provide the first evidence that water-borne steroid conjugates can elicit an endocrine response in a cichlid fish. The increased serum testosterone concentrations induced by the steroid mixture within 45-65 minutes (Figure 4.1) are likely the effect of a rapid increase in serum GtH-II that stimulated steroid hormone production. The functional significance of this testosterone concentration increase remains unclear. However, the observed androgen response implies the role of this steroid mixture as a stimulant of secondary sex characters, reproductive behavior, and/or spermatogenesis (Borg, 1994). It also remains unclear what component(s) of the steroid mixture was responsible for the endocrine change. Five steroids were included in the mixture and it is possible that only one, or a combination of some, elicited the serum testosterone increase.

Attempts to determine the effect of the individual components of the mixture (experiments 2 and 3) were unsuccessful in eliciting a serum testosterone increase

estradiol-3,17-disulfate and 5β-pregnan-3α,17-diol-20-one-glucuronide exposed fish seemed to increase over time, until 120 minutes post odor introduction.

Future attempts to quantify behavioral responses of *H. burtoni* to water-borne steroids should consider the importance of mixtures. It would also be interesting to see various regimes used, perhaps including multiple males in a test aquarium, or fish in adjacent aquaria with only visual contact. This study employed a combination of one male and two females to present males with multiple targets to direct behaviors. Also, males will not successfully mate with females if kept as a pair (T. Cole, personal observation). While adding more fish to a test aquarium can be problematic, additional cues not present in my studies may be required to elicit behavioral responses.

5.3 Endocrine response to steroid hormones

Male serum testosterone concentrations were significantly greater in fish exposed to a mixture of steroid hormones than in controls (Figure 4.1). The mixture of steroids included the most potent representatives of all five proposed olfactory receptor mechanisms. Although the biological function of the testosterone increase in the mixture exposure study is unclear, this result provides valuable and compelling evidence that *H. burtoni* employ a hormonal pheromone system. These results also implicate steroid mixtures as being important factors in this system, as exposure to individual components of this mixture did not result in increased male serum testosterone concentrations (Figure 4.2). However, one component of the steroid mixture (17β-estradiol-17-glucuronide) was not tested individually, and potentially important differences in test procedure (see Chapter 4) may have prevented individually tested steroids from eliciting an endocrine response.

The serum testosterone increase shown by males exposed to a mixture of steroids is likely the effect of a more rapid serum GtH-II increase, which occurs within 15 minutes in goldfish exposed to 17,20β-P (Dulka et al., 1987). We sampled fish at 45-65 minutes post-exposure, and it is possible that these results could have even more pronounced had we sampled fish at a different time. By sampling fish at various times and assaying for several serum steroid concentrations, it would be possible to

develop a detailed model of both endocrine response and steroid metabolism. HPLC and/or EIA should be used to determine what detected steroids are released, when, and by which sex.

5.4 Summary

This research demonstrates that the olfactory organ of *H. burtoni* detects a variety of water-borne steroids, and can discriminate these odors through 5 independent olfactory receptor mechanisms. *H. burtoni* show behavioral responses to some detected steroids, and coupled with the endocrine response seen, these results suggest that *H. burtoni* employs a hormonal pheromone system. The findings of this study may provide an early step in discovering a potential (chemically-mediated) isolating mechanism that has aided in the explosive speciation of cichlids. These results provide a basis to examine the extent of hormonal pheromone use in pair-bonding fish. This research also provides a framework to explore potential fish culture and management techniques that could be useful in cichlid conservation efforts.

5.5 Literature cited

Borg, B. 1994. Androgens in teleost fishes. *Comp. Biochem. Physiol. C.* **109**: 219-245.

Colombo, L., Marconato, A., Belvedere, P.C., and Frisco, C. 1980. Endocrinology of teleost reproduction: A testicular steroid pheromone in the black goby, *Gobius jozo* L. *Boll. Zool.* **47**: 355-364.

Dulka, J.G., Stacey, N.E., Sorensen, P.W., and Van Der Kraak, G.J. 1987. A steroid sex pheromone synchronizes male-female spawning readiness in goldfish. *Nature.* **325**: 251-253.

Frings, S., D. Reuter, S.J. Kleene. 2000. Neuronal Ca^{2+}-activated Cl^- channels: homing in on an elusive channel species. *Prog. Neurobiol.* **60**: 247-289.

Laberge, F. and Hara, T.J. 2001. Neurobiology of fish olfaction: a review. *Brain. Res. Rev.* **36**: 46-59.

Leveteau, J., Andriasson, I., Trotier, D., and Macleod, P. 1989. Role of divalent-

Cations in EOG generation. *Chem. Senses*. **14**: 611-620.

Murphy, C.A., Stacey, N., and Corkum, L.D. 2001. Putative steroidal pheromones in the round goby, *Neogobius melanostomus*: olfactory and behavioral responses. *J. Chem. Ecol.* **27**: 443-470.

Poling, K.R., Fraser, E.J., and Sorensen, P.W. 2001. The three steroidal components of the goldfish preovulatory pheromone signal evoke different behaviors in males. *Comp. Biochem. Physiol. B.* **129**: 645-651.

Resink, J.W., Voorthius, P.K., Van Den Hurk, R., Peters, R.C., and Van Oordt, P.G.W.J. 1989. Steroid glucuronides of the seminal vesicle as olfactory stimuli in African catfish *Clarias gariepinus*. *Aquaculture.* **83**: 153-166.

Scott, A.P. and Sorensen, P.W. 1994. Time course of release of pheromonally active steroids and their conjugates by ovulatory goldfish. *Gen. Comp. Endocrinol.* **96**: 309-323.

Specker, J. L., and Sullivan, C. V. (1994). Vitellogenesis in fishes: status and perspectives. pp 304-315. In *Perspectives in Endocrinology*. Edited by K.G. Davey, R.E. Peter, and S. S. Tobe. National Research Council, Ottawa.

Sorensen, P.W., Scott, A.P., and Kihslinger, R.L. 2000. How common hormonal metabolites function as relatively specific pheromonal signals in goldfish. pp 125-129.In *Proceedings of the Sixth International Symposium on Reproductive Physiology of Fish.* Norberg, B., Kjesbu, O.S., Taranger, G.L., Andersson, E., and Stefansson, S.O., Eds. John Grieg AS, Bergen.

Stacey, N and P.W Sorensen. 2002. Hormonal pheromones in fish. pp 375-434. In *Hormones, Brain, and Behavior volume 2.* Edited by D.W. Pfaff, A.P. Arnold, A.M. Etgen, S.E. Fahrbach and R.T. Rubin. Academic Press, San Diego.

Appendix 1. All steroids and prostaglandins tested (10^{-9} M) in EOG screening of *Haplochromis burtoni*.

All steroids and prostaglandins were purchased from Sigma (St. Louis, MO), Steraloids (Wilton, NJ), Cayman Chemical Company (Ann Arbor, MI), or received as gifts from Dr. Alexander P. Scott (Lowestoft, U.K.)

Table A1.1

Supplier	Catalog number	Chemical name
		5α-androstan free (19C)
Steraloids	A 1630	5α-androstan-3,17-dione
Steraloids	A 2490	5α-androstan-3ß-ol-17-one
Sigma	A 8380	5α-androstan-17ß-ol-3-one
Steraloids	A 1170	5α-androstan-3α,17ß-diol
Steraloids	A 1220	5α-androstan-3ß,17ß-diol
Steraloids	A 1560	5α-androstan-3ß,17ß-diol-11-one
Steraloids	A 2280	5α-androstan-3α-ol-11,17-dione
Steraloids	A 2330	5α-androstan-3ß-ol-11,17-dione
Steraloids	A 2420	5α-androstan-3α-ol-17-one
Steraloids	A 2650	5α-androstan-3-one
Steraloids	A 2720	5α-androstan-17-one
Steraloids	A 2900	5α-androstan-3,11,17-trione
		5α-androstan- glucuronide (19C)
Steraloids	A 1207	5α-androstan-3α-17ß-diol-17-gluc
Sigma	E 8001	5α-androstan-3ß-ol-17-one-3ß-gluc
Sigma	A 7508	5α-androstan-3α-ol-17-one-3α-gluc
Steraloids	A 2510	5α-androstan-3ß-ol-17-one-3-gluc
Steraloids	A 2290	5α-androstan-3α-ol-11,17-dione-3-gluc
Steraloids	A 2430	5α-androstan-3α-ol-17-one-3-gluc
		5α-androstan-sulfate (19C)
Steraloids	A 2460	5α-androstan-3α-ol-17-one-sulfate
Steraloids	A 2534	5α-androstan-3ß-ol-17-one-3-sulfate
		5β-androstan free (19C)
Steraloids	A 3270	5ß-androstan-3,17-dione
Steraloids	A 3670	5ß-androstan-3ß-ol-17-one
Sigma	E 5126	5ß-androstan-3α-ol-17-one

Steraloids	A 3030	5ß-androstan-3α,17ß-diol
Steraloids	A 3060	5ß-androstan-3ß,17ß-diol
Steraloids	A 3120	5ß-androstan-3α,11ß-diol-17-one
Steraloids	A 3260	5ß-androstan-3α,11ß-diol-11-one
Steraloids	A 3460	5ß-androstan-3α-ol-11,17-dione
Steraloids	A 3940	5ß-androstan-3α,11ß,17ß-triol
Steraloids	A 3210	5ß-androstan-3α,17ß-diol-11-one
		5β-androstan-glucuronide (19C)
Steraloids	A 3626	5ß-androstan-3α-ol-17-one-3-gluc
Sigma	E 8000	5ß-androstan-3α-ol-17-one-gluc
Sigma	H 6003	5ß-androstan-3α,11ß-diol-17-one-3-gluc
Scott		5ß-androstan-3α,17ß-diol-17ß-gluc
Scott		5ß-androstan-3α-17β-diol-3α-gluc
Steraloids	A 3120	5ß-androstan-3α-ol-11β-diol-17-one-3-gluc
Steraloids	A 3030	5ß-androstan-3α,17β-diol-3-gluc
		5β-androstan-sulfate (19C)
Steraloids	A 3640	5β-androstan-3α-ol-17-one-3-sulfate
Steraloids	A 3638	5β-androstan-3α-ol-17-one-3-sulfate, K salt
		4-androsten free (19C)
Sigma	A 9630	4-androsten-3,17-dione
Steraloids	A 6630	4-androsten-11ß-ol-3,17-dione
Scott		4-androsten-11-keto-3,17-dione
Sigma	T 1500	4-androsten-17ß-ol-3-one
Sigma	H 4128	4-androsten-11ß-17ß-diol-3-one
Sigma	K 8250	4-androsten-11-keto-17ß-ol-3-one
Sigma	E 5878	4-androsten-17α-ol-3-one
Steraloids	A 6600	4-androsten-11α-ol-3,17-dione
Sigma	N 7252	4-androstren-17ß-ol-3-one
		4-androsten-glucuronide (19C)
Sigma	T 2000	4-androsten-17ß-ol-3-one-17ß-gluc
		4-androsten-sulfate (19C)
Steraloids	A 7010	4-androsten-17β-ol-3-one-sulfate
		5-androsten free (19C)
Steraloids	A 8500	5-androsten-3ß-ol-17-one
Sigma	A 8755	5-androsten-3ß,17ß-diol
Steraloids	A 8501	5-androsten-3ß-ol-17-one-acetate
		5-androsten-glucuronide (19C)
Steraloids	A 8515	5-androsten-3ß-ol-17-one-3-gluc
		5-androsten-sulfate (19C)
Steraloids	A 8530	5-androsten-3ß-ol-17-one-3-sulfate

Steraloids	A 8530	5-androsten-3ß-ol-17-one-sulfate, Na salt
Steraloids	A 8529	5-androsten-3ß-ol-17-one-sulfate, K salt
		estratrienes free (18C)
Sigma	E 9750	1,3,5 (10)-estratrien-3α-ol-17-one
Sigma	E 8875	1,3,5 (10)-estratrien-3α,17ß-diol
Sigma	E 1253	1,3,5 (10)-estratrien-3α,16α,17ß-triol
Steraloids	E 870	1,3,5 (10)-estratrien-3α,17α,diol
		estratrienes glucuronide (18C)
Sigma	E 2127	1,3,5 (10)-estratrien-3α,17ß-diol-3-gluc
Sigma	E 1127	1,3,5 (10)-estratrien-3α,17ß-diol-17-gluc, Na salt
Steraloids	E1073Na	1,3,5 (10)-estratrien-3α,17ß-diol-17-gluc, Na salt
Steraloids	E1072Na	1,3,5 (10)-estratrien-3α,17ß-diol-3-gluc, Na salt
Steraloids	E 1010	1,3,5 (10)-estratrien-3α,17ß-diol-di-gluc
Steraloids	E 2320	1,3,5 (10)-estratrien-3-ol-17-one-gluc
Sigma	E 1752	1,3,5 (10)-estratrien-17-one-3-gluc
		estratrienes-sulfate (18C)
Sigma	E 9505	1,3,5 (10)-estratrien-3α,17ß-diol-3-sulfate
Steraloids	E 1103	1,3,5 (10)-estratrien-3α,17ß-diol-17-sulfate
Steraloids	E 1050	1,3,5 (10)-estratrien-3α,17ß-diol-disulfate
Sigma	E 1636	1,3,5 (10)-estratrien-3a,17ß-diol-disulfate
Steraloids	E 893	1,3,5 (10)-estratrien-3α,17α-diol-3-sulfate, Na salt
Steraloids	E 1100	1,3,5 (10)-estratrien-3α,17ß-diol-3-sulfate, Na salt
Steraloids	E 2335	1,3,5 (10)-estratrien-3-ol-17-one-sulfate, Na salt
		estratrienes-glucurinide-sulfate (18C)
Sigma	E 2128	1,3,5 (10)-estratrien-3-gluc-17-sulfate
Sigma	E 3890	1,3,5 (10)-estratrien-3-sulfate-17-gluc
		5α-pregnan free (C21)
Steraloids	P 2750	5α-pregnan-3,20-dione
Steraloids	P 3700	5α-pregnan-17-ol-3,20-dione
Steraloids	P 2320	5α-pregnan-17,21-diol-3,20-dione
Steraloids	P 5250	5α-pregnan-11ß,17,21-triol-3,20-dione
Steraloids	P 5000	5α-pregnan-3α,17,20ß-triol
Steraloids	P 5450	5α-pregnan-3α,17,21-triol-20-one
Steraloids	P 3830	5α-pregnan-3ß-ol-20-one
Steraloids	P 2490	5α-pregnan-3ß,17-diol-20-one
Steraloids	P 2050	5α-pregnan-3ß,20α-diol
Steraloids	P 2100	5α-pregnan-3ß,20ß-diol
Sigma	P 0773	5a-pregnan-3ß,17,20ß-triol
Steraloids	P 5500	5a-pregnan-3ß,17,21-triol-20-one
Steraloids	P 4850	5a-pregnan-3ß,11ß,17,21-tetrol-20-one
		5α-pregnan-sulfate (C21)
Steraloids	P 2135	5α-pregnan-3ß,20ß-diol-3-sulfate, Na salt

Steraloids	P 3817	5α-pregnan-3α-ol-20-one-sulfate, Na salt
Steraloids	P 3865	5α-pregnan-3ß-ol-20-one-sulfate, Na salt
		5β-pregnan free (C21)
Steraloids	P 7150	5ß-pregnan-3,20-dione
Steraloids	P 6300	5ß-pregnan-17,21-diol-3,20-dione
Steraloids	P 8090	5ß-pregnan-17α-ol-3,20-dione
Steraloids	P 9650	5ß-pregnan-11ß,17,21-triol-3,20-dione
Steraloids	P 6050	5ß-pregnan-3α,20ß-diol
Steraloids	P 6570	5ß-pregnan-3α,17-diol-20-one
Steraloids	P 8990	5ß-pregnan-3α-17α,20α,21-tetrol
Sigma	P 8629	5ß-pregnan-3α,17α,20α-triol
Sigma	P 8258	5ß-pregnan-3α,17α,20ß-triol
Steraloids	P 9000	5ß-pregnan-3α,17,20ß,21-tetrol
Steraloids	P 9050	5ß-pregnan-3α,11ß,17,21-tetrol-20-one
Steraloids	P 8540	5ß-pregnan-3α,11ß,17α,20α,21-pentol
Steraloids	P 8590	5ß-pregnan-3α,11ß,17α,20ß,21-pentol
Steraloids	P 9200	5ß-pregnan-3α,17,20ß,21-tetrol-11-one
Steraloids	P 9550	5ß-pregnan-3α,17,21-triol-11,20-dione
Steraloids	Q 200	5ß-pregnan-3α,17,21-triol-20-one
Steraloids	P 6140	5ß-pregnan-3ß,20ß-diol
Steraloids	P 6810	5ß-pregnan-3ß,17-diol-20-one
Steraloids	Q 250	5ß-pregnan-3ß,17,21-triol-20-one
Sigma	P 1648	5ß-pregnan-3ß,11ß,17,21-tetrol-20-one
		5β-pregnan-glucuronide (C21)
Steraloids	P 9580	5ß-pregnan-3α-17,21-triol-11,20-dione-3-gluc
Steraloids	P 6587	5ß-pregnan-3α,17-diol-20-one-gluc
		5β-pregnan-sulfate (C21)
Steraloids	P 8168	5ß-pregnan-3α-ol-20-one-sulfate, Na salt
		4-pregnen free (C21)
Sigma	P 0130	4-pregnen-3,20-dione
Steraloids	Q 2600	4-pregnen-3,20-dione
Sigma	H 5502	4-pregnen-11α-ol-3,20-dione
Sigma	H 5627	4-pregnen-11ß-ol-3,20-dione
Sigma	H 5752	4-pregnen-17α-ol-3,20-dione
Steraloids	Q 3360	4-pregnen-17α-ol-3,20-dione
Sigma	P 6288	4-pregnen-20ß-ol-3-one
Sigma	P 6163	4-pregnen-20ß-ol-3-one
Steraloids	Q 3630	4-pregnen-20ß-ol-3-one
Steraloids	Q 3600	4-pregnen-20α-ol-3-one
Sigma	P 6288	4-pregnen-20α-ol-3-one
Steraloids	Q 3600	4-pregnen-20α-ol-3-one
Sigma	P 6285	4-pregnen-17α,20ß-diol-3-one
Sigma	P 6160	4-pregnen-17α,20a-diol-3-one

Sigma	R 0500	4-pregnen-17,21-diol-3,20-dione
Steraloids	Q 1970	4-pregnen-20ß,21-diol-3-one
Sigma	D 6875	4-pregnen-21-ol-3,20-dione
Steraloids	Q 4080	4-pregnen-17,20ß,21-triol-3-one
Sigma	P 9647	4-pregnen-17,20α,21-triol-3-one
Steraloids	Q 3880	4-pregnen-11ß,17,21-triol-3,20-dione
Sigma	P 9129	4-pregnen-11-keto-17,21-diol-3,20-dione
Steraloids	Q 3960	4-pregnen-17,20ß,21-triol-3,11-dione
Steraloids	Q 3580	4-pregnen-17α-ol-3-one
Steraloids	Q 1550	4-pregnen-11ß,21-diol-3,20-dione
Steraloids	Q 1580	4-pregnen-16α,17ß-diol-3,20-dione
Steraloids	Q 3920	4-pregnen-14α,17ß,21-triol-3,20-dione
Steraloids	Q 4160	4-pregnen-3,11,20-trione
Steraloids	Q 2500	4-pregnen-17,21-diol-3,11,20-trione
Steraloids	Q 3790	4-pregnen-11ß,17,20ß,21-tetrol-3-one
Steraloids	Q-3960	4-pregnen-17,20ß,21-triol-3,11-dione
Steraloids	Q 3850	4-pregnen-11α,17,21-triol-3,20-dione
Sigma	P 9521	4-pregnen-11ß,17α-diol-3,20-dione
		4-pregnen-glucuronide (C21)
Steraloids	Q 1865	4-pregnen-17α,20ß-diol-3,20-dione-20-gluc
Steraloids	Q 3470	4-pregnen-21-ol-3,20-dione-21-gluc
Steraloids	Q 3890	4-pregnen-11ß,17,21-triol-3,20-dione-21-gluc
		4-pregnen-sulfate (C21)
Scott		4-pregnen-20ß-ol-3-one-20-sulfate
Steraloids	Q 1890	4-pregnen-17α,20ß-diol-3-one-20ß-sulfate
Scott		4-pregnen-17α,20a-diol-3-one-20α-sulafte
Steraloids	Q 1625	4-pregnen-17,21-diol-3,20-dione-21-sulfate
Scott		4-pregnen-17,20ß,21-triol-3-one-sulfate
Steraloids	Q 3910	4-pregnen-11ß,17,21-triol-3,20-dione-sulfate
Sigma	C 6162	4-pregnen-11ß-21-diol-3,20-dione-21-sulfate
Steraloids	Q 3383	4-pregnen-17-ol-3,20-dione-sulfate
		5-pregnen free (C21)
Steraloids	Q 4780	5-pregnen-3ß,21-diol-20-one
Steraloids	Q 5900	5-pregnen-3ß,17,20ß-triol
Steraloids	Q 6050	5-pregnen-3ß,17,21-triol-20-one
Sigma	P 9129	5-pregnen-3ß-ol-20-one
Sigma	H 5002	5-pregnen-3ß,17-diol-20-one
Steraloids	Q 4470	5-pregnen-3ß,20ß-diol-11-one
		5-pregnen-glucuronide (C21)
Steraloids	Q 5520	5-pregnen-3ß-ol-20-one-3-gluc
		5-pregnen-sulfate (C21)
Steraloids	Q 4765	5-pregnen-3ß,17-diol-20-one-3-sulfate
Steraloids	Q 4800	5-pregnen-3ß,21-diol-20-one-21-sulfate

Steraloids	Q 5545	5-pregnen-3ß-ol-20-one-3-sulfate
		Prostaglandins
Cayman	13010	Prostaglandin E1
Cayman	14010	Prostaglandin E2
Cayman	15010	Prostaglandin F1α
Cayman	16010	Prostaglandin F2α
Cayman	16990	Prostaglandin F3α
Cayman	14720	15-keto-Prostaglandin E2
Cayman	16720	15-keto-Prostaglandin F2α
Cayman	16670	13,14-dihydro-15-keto-Prostaglandin F2α

Appendix 2. EOG response profiles and their influence on binary mixture experiment analysis.

EOG responses are all generally similar, consisting of a rapid rising phase followed by a slower decline. Response latencies can vary, from 32 msec in the rat (Mackay-Sim and Kesteven, 1994) to between 100-200 msec in the salamander (Van As et al.,1985). However, no studies have examined differences in response profiles to different odorants. This is presumably because the majority of EOG measurements focus only on the peak amplitude of the response, or in some cases the areas under the response curves. In the case of binary mixture experiments, slight variations in EOG response profiles between two stimuli (i.e. temporal shifts in response peaks) can have significant effects on subsequent analysis. Current binary mixture analysis examines the additive effect of two odors through calculations that assume the response of a mixture will be equal to the sum of the responses generated by each of the independent components. If the components do not induce peak amplitude at the same time, this criterion cannot be met.

Figure A2.1 shows a sample of EOG response curves from three test odors, L-alanine (10^{-5} M), etiocholanolone-glucuronide (10^{-8} M), and 17β-estradiol-17-glucuronide (10^{-8} M). Each odor elicits a different response curve, with peak magnitudes occurring at slightly different times. Figure A2.2 shows traces from an actual binary mixture test using two odors known to act through separate receptor mechanisms: an amino acid L-alanine (10^{-5} M) and a steroid 17β-estradiol-17-glucuronide (10^{-8} M). Independently, L-alanine and 17β-estradiol-17-glucuronide elicit EOG responses of 9.64 mV and 3.34 mV, respectively. When tested as a mixture they should elicit an EOG response of 12.98 mV, in order to produce an ICI value of 1, which would suggest that these two odors act via separate receptor mechanisms (see chapter 2 for details). However, when tested as a mixture these compounds elicit an EOG response of 12.4, resulting in an ICI value of 0.95, suggesting some lack of independence. Although this difference appears minor, it can certainly affect the outcome of binary mixture analysis, and has the potential to cloud the relationships between detected odors.

LITERATURE CITED

Mackay-Sim, A. and Kesteven, S. 1994. Topographic patterns of responsiveness to odorants in the rat olfactory epithelium. *J. Neurophysiol.* **71**: 150-160.

Van As, W., J.S. Kauer, B.P.M. Menco, and E.P. Koster. 1985. Quantitative aspects of the electro-olfactogram in the tiger salamander. *Chem. Senses.* **10**: 1-21.

Figure A2.1. Sample EOG response curves from three test odors, L-alanine (10^{-5} M) (solid line); etiocholanolone-glucuronide (10^{-8} M) (dashed line); and 17β-estradiol-17-glucuronide (10^{-8} M) (dotted line).

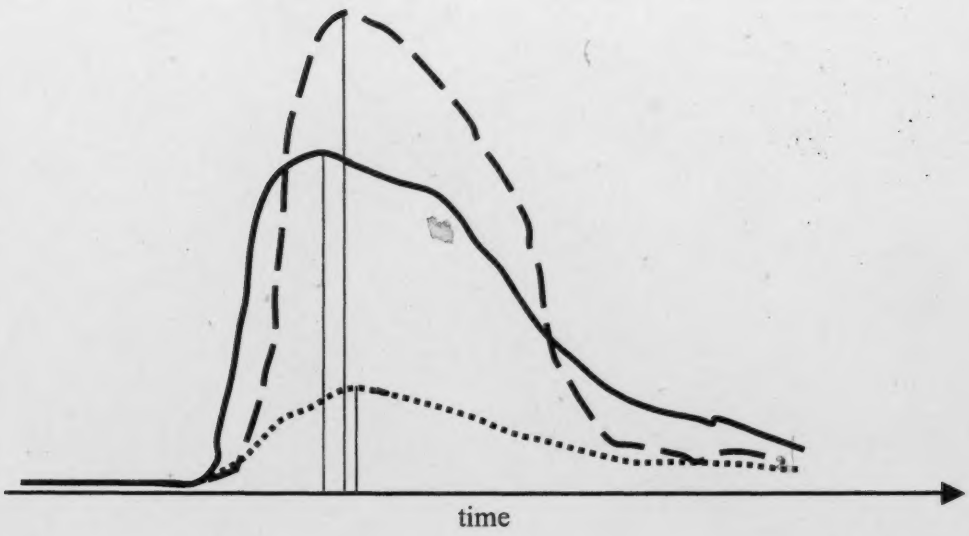
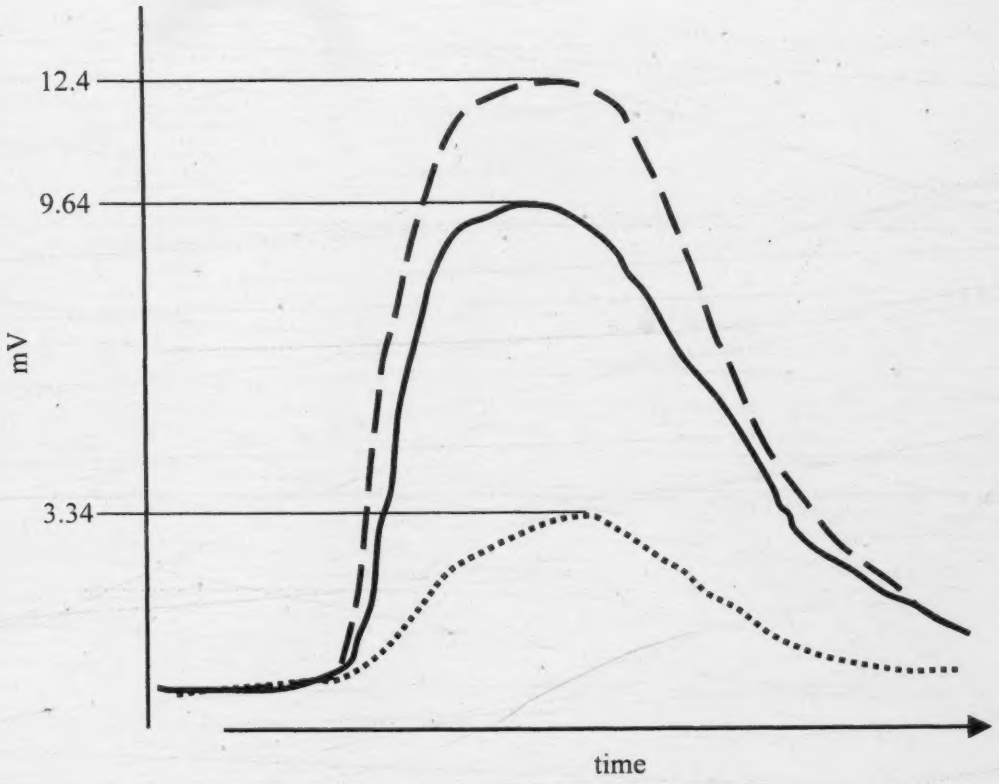

time

Figure A2.2. EOG response (mV) curves from a binary mixture test using L-alanine (10^{-5} M) (solid line) and 17β-estradiol-17-glucuronide (10^{-8} M) (dotted line). The response of the mixture (dashed line) does not equal the sum of the individual responses.


12.4
9.64
3.34
mV
time